PKCζ als neues Effektormolekül des EGF Rezeptors: Aktivierung durch Protein-Protein Interaktion und Phosphorylierung an Threonin 410 und Tyrosin 417

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PKCζ as a novel effector molecule of EGFR: catalytic activation by protein-protein interaction involves threonine 410 and tyrosine 417 phosphorylation

Ph.D. thesis

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by Christina Valkova, M.Sc.
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dedicated to my parents

ZUSAMMENFASSUNG

Die verschiedenen Isoformen der Protein stellt offenbar einen zusätzlichen Mechanismus zur Regulation der Aktivität und Funktion Kinase C (PKC) Superfamilie sind gemeinsame Signalproteine sowohl von G Protein-gekoppelten Rezeptoren (GPCRs) als auch von Rezeptortyrosinkinasen. Damit erhalten sie eine Schlüsselfunktion im cross-talk dieser Rezeptoren und im zellulären Netzwerk der Signaltransduktion. Die Aktivierung verschiedener PKC Isoformen vermittelt unterschiedliche zelluläre Reaktionen, wie z. B. Zellwachstum, subzelluläre Lokalisierung, Lipid-Protein oder Protein-Protein-Interaktionen. Wie kürzlich festgestellt wurde können verschieden PKC Isoformen durch unterschiedliche Stimuli auch an Tyrosinresten phosphoryliert werden. Dies stellt einen weiteren Mechanismus zur Regulation der Aktivität und Funktion von PKCs dar.
Ziel der vorliegenden Arbeit ist die Untersuchung der Tyrosin-Phosphorylierung von PKC Isoformen nach Stimulierung des EGF Rezeoptors (EGFR). Bisher wurde in der Literatur lediglich über eine EGFR-vermittelte Tyrosin-Phosphorylierung und dadurch Inaktivierung von PKCδ berichtet, die eine Rolle bei der neoplastischen Transformation von Keratinozyten zu spielen scheint (Denning *et al., 1996).* In unserem zellulären Modell COS-7 Zelle konnten wir die Tyrosin-Phosphorylierung vonPKCδ durch EGF reproduzieren und fanden zusätzlich eine EGF-induzierte Tyrosin-Phosphorylierung der n(novel) PKCε und der a(atypical) PKCζ. Die weiteren Untersuchungen dieser Arbeit konzentrierten sich ausschließlich auf die aPKCζ. Für PKCζ ist eine Beteiligung an der Regulation von proliferativen, apoptotischen als auch anti-apoptotischen Prozessen bekannt. Weiterhin wurde beschrieben, dass PKCζ mit Hilfe von Adaptorproteinen an verschiedene Rezeptorkomplexe (z.B. TNFR, IL-IR, IR, NGFR) oder K+-Kanäle rekrutiert werden kann.

Wir konnten eine schnelle und Konzentrations-abhängige Tyrosin-Phosphorylierung von PKCζ durch den Wachstumsfaktor EGF nachweisen. Diese ist abhängig von den Tyrosinkinase-Aktivitäten des EGFR und der zytosolischen Tyrosinkinase Src, der Src-spezifischen Phosphorylierung des EGFR-Tyrosinrestes 845, und der Aktivität einer PI-3 Kinase. Wir fanden weiterhin eine PKC-vermittelte Threonin-Phosphorlyierung des EGFR und eine reverse Korrelation von PKC-Aktivität und deren Tyrosin-Phosphorylierung. Dies deutet darauf hin, dass die EGFR-vermittelte Regulation der PKCζ durch die wechselseitige Phosphorylierung von EGFR und PKCζ kontrolliert wird.

Nach Stimulierung des EGFR transloziert die PKCζ an die Zellmembran und wird dort katalytisch aktiviert. Mittels Ko-Immunopräzipitierung und Ko-Lokalisierung können wir zeigen, dass die EGFR-vermittelte Tyrosin-Phosphorylierung und dadurch Aktivierung der PKCζ die physikalische Assoziation beider Proteine einschließt. Wir können ferner nachweisen,

dass auch eine Transaktivierung des EGFR über G Protein-gekoppelte Rezeptoren, wie z.B. den LPAR und den beta2-AR, die Interaktion zwischen EGFR und PKCζ sowie deren Tyrosin-Phosphorylierung und Aktivierung auslösen kann.

Durch gezielte Mutagenese können wir zeigen, dass der Tyrosinrest 417 von PKCζ eine Schlüsselstellung bei der EGFR-vermittelten Aktivierung der PKCζ besitzt. Tyrosin 417 befindet sich in unmittelbarer Nähe von Threonin 410 im Aktivierungsloop der PKCζ. Dessen Phosphorylierung durch die Proteinkinase PDK-1 ist ein essentieller Schritt der katalytischen Aktivierung von PKCζ. Substitution sowohl von Thr 410 durch Ala als auch von Tyr 417 durch Phe führt dazu, dass PKCζ nicht mehr durch EGF aktiviert werden kann. Wir postulieren deshalb, dass die Phosphorylierung beider benachbarter Aminosäuren sowohl für die basale Aktivität als auch die EGF-induzierte Aktivierung der PKCζ notwendig ist.
ERK1/2, NF-κB und PKB/Akt wurden als Targetproteine der PKCζ und der Signaltransduktion des EGFR identifiziert. Überexpression von PKCζ resultiert in einer Erhöhung der EGF-induzierten Aktvierung von ERK1/2 und der Phosphorylierung von IκBα. Dies ist ein essentieller Zwischenschritt der Aktivierung von NF-κB. Beide Effekte treten nicht auf, wenn die Mutante PKCζ-Y147F überexpremiert wird. Im Gegensatz dazu wird die Phosphorylierung und dadurch Aktivierung von PKB/Akt nicht signifikant durch die Überexpression von Wildtyp-PKCζ beeinflusst, bei Überexpression der PKCζ-Y417F Mutante jedoch signifikant reduziert. Daraus kann geschlussfolgert werden, dass die EGFR-vermittelte Tyrosin-Phosphorylierung von PKCζ entweder durch die katalytische Aktivierung der PKCζ oder durch die Generierung von Phosphotyrosin-Bindungsstellen in die Regulation von Signalwegen der Proliferation und des zellulären Überlebens, wie z.B. die Aktivierung von ERK1/2, NF-κB und PKB/Akt eingebunden ist. Diese Hypothese wird unterstützt durch unsere Befunde, dass die basale DNA Synthese durch die Überexpression von PKCζ Wildtyp, nicht aber durch Überexpression der PKCζ-Y417F Mutante erhöht wird.

Die hier vorgestellten Ergebnisse identifizieren die PKCζ als neuen Effektor des EGFR. Ihre Aktivierung durch Tyrosinphosphorylierung setzt die physikalische Assoziation mit dem EGFR-Signalkomplex voraus und ist abhängig von der cytosolische Tyrosinkinase Src sowie der Src-spezifischen Phosphorylierungsstelle Y845 des EGFR. Damit wurde auch ein bisher unbekanntes Beispiel für den Synergismus von Src und EGFR bei der Regulation des Zellwachstums gefunden. Wir konnten zeigen, dass die EGFR-vermittelte Aktivierung der PKCζ nicht nur den bisher bekannten Aktivierungsmechanismus der PDK-1-abhängigen Phosphorylierung an Threonin 410 im Aktivierungsloop einschließt, sondern zusätzlich der Tyrosinrest 417 in unmittelbarer Nähe des Aktivierungsloops phosphoryliert werden muß. Diese Tyrosinphosphorylierung der PKCζ ist notwendig für deren proliferative und anti-apoptotische Aktivitäten.

SUMMARY

Protein kinase C (PKC) family of serine/threonine protein kinases represents a key mediator of a variety of signalling pathways, including those of G protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs) and their cross talk. Activation of distinct PKC isoforms is implicated in diverse cellular responses such as proliferation, cell cycle control, differentiation, tumorigenesis, cell migration, survival and apoptosis. PKC activity and function are highly regulated by serine/threonine phosphorylation, subcellular targeting, and lipid-protein and protein-protein interactions. Recently, tyrosine phosphorylation of several PKC isoforms in response to various stimuli has been identified as an additional mechanism to regulate PKC activity and function.

The current work was aimed on investigation of the tyrosine phosphorylation of PKC isoforms upon EGFR stimulation. So far, EGFR-mediated tyrosine phosphorylation and inactivation of PKCδ has been reported as an important element of the neoplastic transformation in keratinocytes (Denning *et al*., 1996). We identified that in addition to PKCδ PKCε and PKCζ are also tyrosine phosphorylated in response to EGF stimulation in COS-7 cells. This work is focused on EGFR-mediated tyrosine phosphorylation of PKCζ. Protein kinase C zeta is an atypical PKC, which is involved in proliferative and pro-survival as well as anti-survival signalling and is recruited via adaptor proteins to many receptor complexes (TNFR, IL-1R, IR, NGFR) and to the K^+ channels.

The rapid tyrosine phosphorylation of PKCζ downstream of EGFR was found to be strongly dependent on EGFR and Src tyrosine kinase activities, on Src-phosphorylation site of EGFR tyrosine 845, and partly dependent on PI3K activity. We observed threonine phosphorylation of EGFR upon stimulation and a reverse correlation between PKCζ activity state and its level of tyrosine phosphorylation. This indicates indirectly that reciprocal phosphorylation between EGFR and PKCζ might control EGFR-mediated modification of PKCζ.

In response to EGFR stimulation PKCζ translocates to the plasma membrane and is catalytically activated. Using co-immunoprecipitation and co-localization studies we identified that EGFR-mediated tyrosine phosphorylation and activation of PKCζ involves physical association of PKCζ with EGFR. Furthermore, EGFR transactivation upon stimulation of GPCRs (LPAR and β2AR) led also to tyrosine phosphorylation and activation of PKCζ accompanied by interaction of PKCζ with the EGFR.

We identified tyrosine 417 of PKCζ as one major EGFR-dependent phosphorylation site, critically implicated in EGFR-mediated activation of PKCζ. Tyrosine 417 is located in the vicinity of threonine 410 at the activation loop of PKCζ, whose phosphorylation by PDK-1 is known to be necessary for PKCζ catalytic activation. Substitution of either threonine 410 to

alanine or tyrosine 417 to phenylalanine renders PKCζ unresponsive to activation by EGF. Thus, both threonine phosphorylation at the activation loop and tyrosine phosphorylation in its vicinity are required for the basal activity and for EGFR-mediated catalytic activation of PKCζ.

ERK1/2, NF-κB and PKB/Akt activation were identified as targets of PKCζ in EGFR downstream signalling. Overexpression of PKCζ wild type caused increase in EGF-induced ERK1/2 activity and in IκBα phosphorylation, which is a prerequisite for NF-κB activation. Both effects are lost upon expression of PKCζ Y417F. On the other hand, PKB/Akt phosphorylation indicative for its activation- was not significantly affected by overexpression of PKCζ but is significantly reduced upon expression of PKCζ Y417F. Thus, EGFR-mediated tyrosine phosphorylation of PKCζ is implicated either through PKCζ catalytic activation or via creation of phosphotyrosine-binding sites in proliferative and pro-survival signalling downstream of EGFR through Erk1/2, NF-κB and PKB/Akt activation. Supporting evidence that PKCζ pro-proliferative role is provided by its tyrosine phosphorylation was the increased basal DNA synthesis upon overexpression of PKCζ wild type, but not of PKCζ Y417F.

Taken together, we identified PKCζ tyrosine phosphorylation upon EGFR stimulation as a novel effector of the synergism between EGFR and Src in the regulation of cell proliferation and survival. Moreover, physical association between EGFR and PKCζ has been identified. EGFR-mediated activation of PKCζ within the EGFR signalling complex involves not only PDK1-dependent phosphorylation of threonine 410 at the activation loop, but also requires phosphorylation of tyrosine 417 in activation loop vicinity. PKCζ tyrosine phosphorylation is necessary for its pro-proliferative and pro-survival function.

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1. INTRODUCTION

Plasma membrane-spanning receptors receive extracellular signals and transmit them into specific cellular responses through activation of several signalling cascades. Both receptor tyrosine kinases (RTK) as well as G protein-coupled receptors (GPCRs) can activate the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) cascade, composed of three sequentially activated by phosphorylation kinases and thereby induce cell proliferation. Individual signalling pathways do not function in isolation but are interconnected into cellular networks and their extensive cross talk may result either in signal integration and thereby amplification /attenuation or signal diversification. The complex interactions between GPCR and RTK signalling pathways towards ERK/ MAPK activation are example for such a cross talk, which is implicated in normal cellular as well as in pathophysiological processes (e.g. cancer, cardiac hypertrophy and cystic fibrosis). In addition to the generation of second messengers intracellular signal transduction is mediated by reversible regulation of protein function by phosphorylation on serine, threonine and tyrosine residues. Protein phosphorylation can control the enzymatic activity, the protein-protein interactions, subcellular localization and susceptibility for protease degradation. Members of protein kinase C (PKC) family of serine/threonine protein kinases are involved in both GPCR and RTK signalling. PKC regulation by tyrosine phosphorylation might contribute to the cross talk mechanisms operating between GPCRs and RTKs.

1.1. Epidermal growth factor receptor (EGFR) signalling

RTKs are family of membrane receptors with intrinsic tyrosine kinase activity that transmit signals promoting cell proliferation, differentiation, migration and modulation of apoptosis. Epidermal growth factor receptor (EGFR, ErbB1, HER1) as a prototypical RTK represents a 170kDa polypeptide consisting of an extracellular ligand-binding domain, a single transmembrane domain and an intracellular domain, harboring the conserved tyrosine kinase subdomain. Eight EGFR ligands are described so far-epidermal growth factor (EGF), heparin-binding epidermal growth factor (HB-EGF), amphiregulin, transforming growth factor alpha (TGFα), betacellulin, epiregulin, epigen and crypto (Fischer *et al*., 2003). Ligand binding to the EGFR extracellular domain promotes dimerization of two receptor molecules, activation of intrinsic tyrosine kinase activity and subsequent autophosphorylation in trans of set of tyrosine residues residing in the intracellular part of the receptor (Schlessinger, 2000) (Figure 1.1.). Thereby docking sites for enzymes and adaptor molecules containing SH2 and PTB domains are created and a set of signalling pathways is activated (Figure 1.2.). One of the major pathways activated downstream of EGFR is the ERK/MAPK cascade initiated by binding of the adaptor

protein Grb2 to the activated receptor directly or indirectly (via Shc) and translocation of guanine nucleotide exchange factor Sos close to Ras at the plasma membrane. The active GTP-bound Ras activates Raf, which, in turn, phosphorylates and activates MEK (MAPK kinase). MEK phosphorylates MAPK (ERK) on Thr and Tyr residues at the activation loop leading to its activation. Activated MAPK phosphorylates multiple cytoplasmic substrates and translocates to the nucleus where it phosphorylates and activates transcription factors.

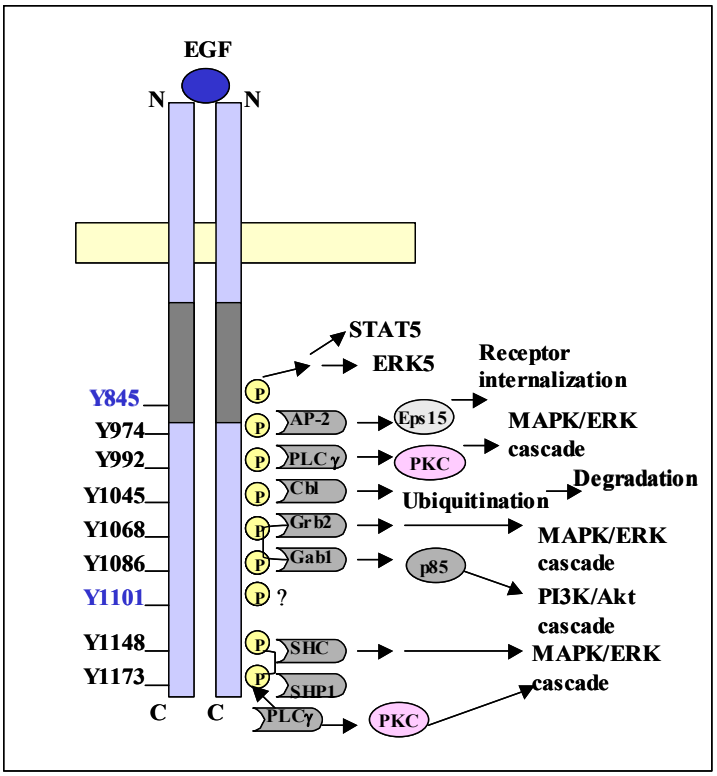


Figure 1.1. Binding of the docking proteins to the specific phosphotyrosine residue in the cytoplasmic part of the activated EGFR receptor. Presented are the autophosphorylation sites (in black) as well as Src-phosphorylation sites (in blue). Shown are the respective downstream signalling cascades and pathways involved in receptor internalization and degradation. Adapted from Cell Signaling catalog, 2003-2004.

Phospholipase C gamma (PLCγ) binds directly to the activated receptor and is activated by tyrosine phosphorylation. Activated PLCγ hydrolyses PtdIns(4,5)P_2 and form two second messengers, diacylglycerol (DAG), a potent activator of protein kinase C (PKC) and Ins(1,4,5)P_3, which stimulates release of intracellular Ca^{2+}. Phosphoinositide 3-kinase (PI3K) is activated upon binding of the regulatory subunit p85 to the adaptor protein Gab1 and leads to generation of second messengers PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (PIP3), thereby mediating membrane translocation and activation via PDK1 of pro-survival kinase PKB /Akt. STATs, JNK and BMK1/ERK5 are also activated downstream of EGFR activation (Kloth *et al*., 2003; Logan *et al*., 1997; Kato *et al*., 1998). EGFR activity is under tight autoinhibitory control via recruitment of protein-tyrosine phosphatases (PTPs) and dephosphorylation of regulatory phospho-tyrosine residues, process that is counteracted by receptor-induced H_2O_2 generation and PTP inactivation (Oestman and Boehmer, 2001). PKC phosphorylates EGFR in the

juxtamembrane region leading to receptor desensitization (Cochet *et al*., 1984). Attenuation and /or termination of EGFR signalling also involve either endocytosis of the receptor and its lysosomal degradation mediated by Eps or proteosomal degradation mediated by Cbl – dependent ubiquitination (Liebmann and Boehmer, 2000). Recently, considerable evidence that EGFR continues to signal in the endosomal compartment has been presented (Hough *et al*., 2002).

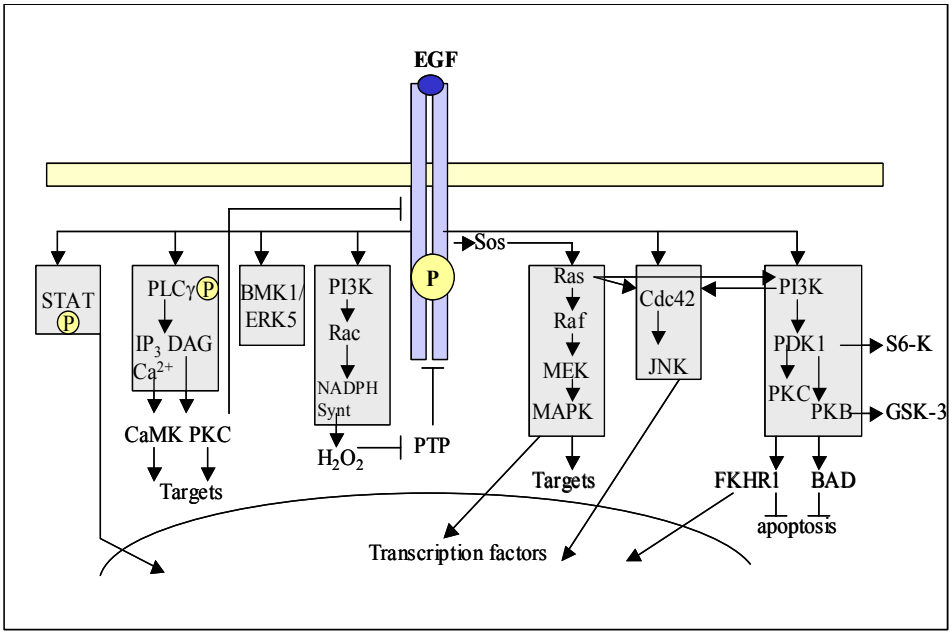


Figure 1.2. Signalling pathways activated by EGFR. Shown is the activation of MAPK, JNK, PI3K/PKB, PLCγ ERK5, STAT and the pathway leading to hydrogen peroxide generation. Adapted from Schlessinger, 2000.

1.2. Cross talk between G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs)

GPCR family of seven-transmembrane domain receptors transduces a huge variety of extracellular signals via Gα and Gβγ subunits of heterotrimeric G-proteins to several effectors like adenylate cyclase (AC), PLCβ, PI3K, PKC, PLD, PLA_2, Ca^{2+} and K^+ channels (Liebmann and Boehmer, 2000). In addition to their involvement in many tissue-specific, competely differentiated cell functions GPCRs can elicit mitogenic responses as well via activation of ERK/MAPK cascade, which is originally employed in the RTK mitogenic signalling (Gutkind, 1998). Diverse pathways link GPCR signalling to ERK/MAPK activation (Figure 1.3.). GPCR-induced RTK transactivation might involve stimulation of membrane-bound metalloproteinases (MP), which, in turn, induce extracellular release of EGF-like ligands from their latent, plasma membrane-spanning precursors as well as pathways that do not involve EGF-like ligand release. Moreover, GPCR-mediated activation of the ERK/MAPK cascade can take place by a number of alternative mechanisms independently of RTK activation (Wetzker and Boehmer, 2003). RTKs can also modulate the activity of signalling pathways, traditionally thought to be

controlled exclusively by GPCRs – for example AC activation upon Gαs activation induced by EGFR (Poppleton *et al.,* 1996). Many aspects of the cross talk between GPCR and RTK involve tyrosine phosphorylation: GPCR-induced tyrosine phosphorylation of RTKs (transactivation), GPCR- and RTK-induced tyrosine phopshorylation of Gα subunits, homologous (ligand-induced), and heterologous (RTK-induced) tyrosine phosphorylation of GPCRs (Liebmann and Boehmer, 2000). For example, EGFR-induced tyrosine phosphorylation of Gαs leads to the loss of its susceptibility to GPCRs (Liebmann *et al*., 1996). PKC isoforms are activated downstream of both GPCRs and RTKs via classical DAG-dependent pathway through activation of PLCβ/γ and PLD or DAG-independent via PI3K activation.

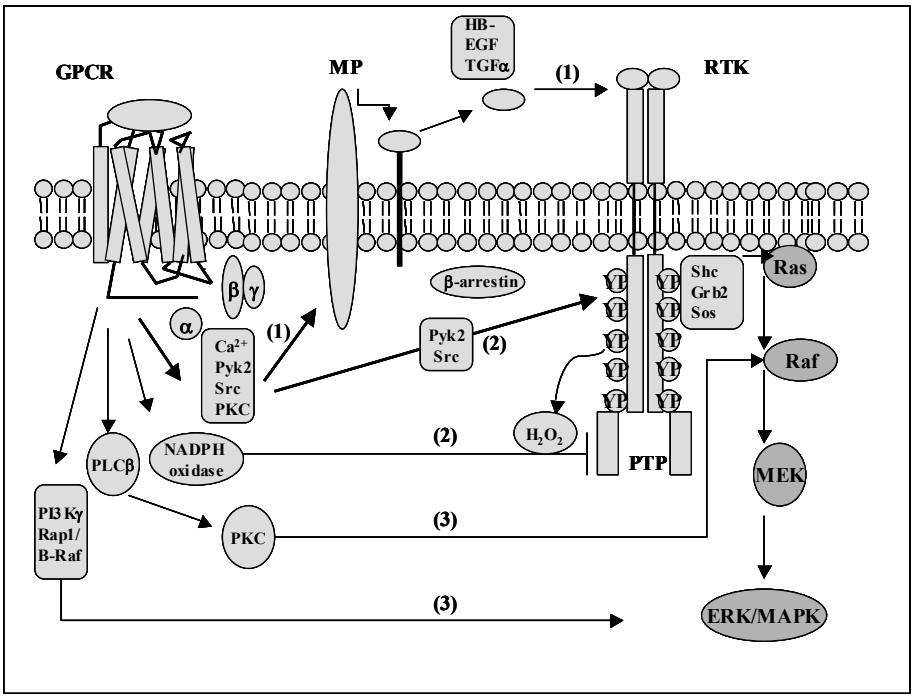


Figure 1.3. Pathways that couple GPCR signalling to RTK and ERK/MAPK activation. Presented are the RTK transactivation-involving pathways-with (1) or without EGF-like ligand release (2) as well as RTK-independent activation of ERK/MAPK by GPCRs (3) and the intermediate signalling proteins. Adapted from Wetzker and Boehmer, 2003.

A role of PKC isoforms in EGFR transactivation upstream (Shah and Catt, 2002) or downstream of the receptor (Buteau *et al*., 2003) as well as in EGFR-independent ERK/MAPK activation by GPCRs (Adomeit *et al*., 1999) has been demonstrated. Tyrosine phosphorylation of PKC isoforms by RTKs directly stimulated by growth factors or transactivated by GPCRs has been suggested to bring about additional level of complexity and functional specificity in cellular signalling (Liebmann and Boehmer, 2000).

1.3. Protein kinase C –family members, domain structure and regulation

1.3.1. PKC family members and domain structure

PKC family of serine/threonine protein kinases represents a key player in a variety of cellular processes including proliferation, differentiation, apoptosis, survival and tumorigenesis. The mammalian protein kinase C family comprises 10 isozymes grouped into three subfamilies based on their primary structure and cofactor dependences: conventional or classical PKC (α, alternatively spliced βI and βII and γ), novel PKC (δ, ε, η, θ) and atypical PKC (ζ, ι/λ). PKCμ/D and ν (PKD) and PKC-related kinases (PRK) are considered as more distantly related to PKC family enzymes with some unique features (Figure 1.4.). The activity of classical isoforms (cPKCs) is stimulated by cofactors diacylglycerol (DAG), $Ca^{2+}$and phosphatidylserine (PtdSer). Novel PKC isoforms (nPKCs) are Ca^{2+}-independent, but stimulated by DAG and PtdSer, whereas atypical PKCs (aPKCs) are $Ca^{2+}$and DAG unresponsive, but stimulated by PtdSer. The single polypeptide chain of all PKC isozymes consists of carboxy-terminal kinase domain and amino-terminal regulatory moiety, connected via proteolitically labile 'hinge'region. Both domains contain conserved (C) regions of extended sequence homology and variable (V) regions. As a multidomain protein PKC is under acute lipid and protein-mediated conformational regulation of interdomain interactions (Newton, 2003). The regulatory moiety consists of pseudosubstrate (PS) and two membrane targeting modules- C1 and C2 domains. PS resembles the substrate except of harboring alanine at the phosphoacceptor position and occupies the substrate-binding cavity of the catalytic domain thereby keeping the enzyme in an autoinhibited conformation. The C1 domain present in all PKC isoforms is a Cys-rich region that binds the second messenger of phospholipid metabolism DAG and tumor-promoting phorbol esters (PE) and in ligand-bound form confers specific binding to PtdSer in the membrane.

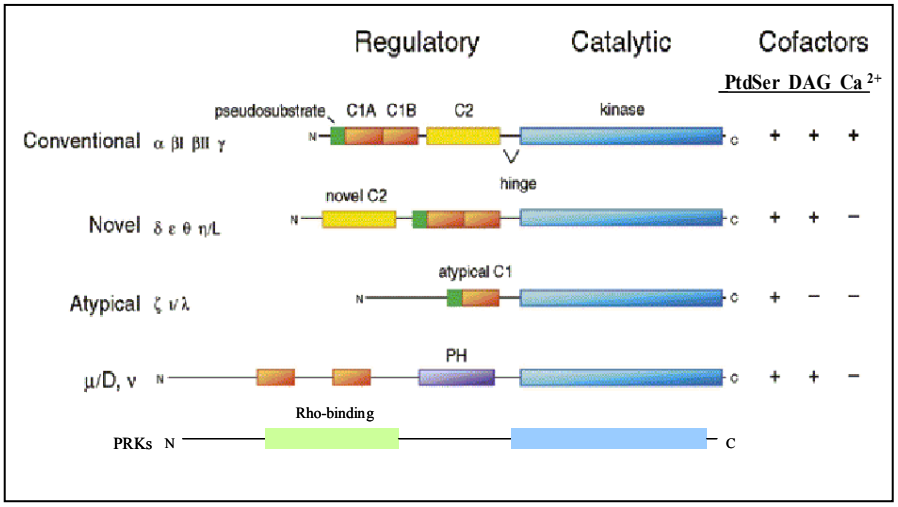


Figure 1.4. Protein kinase C family members- domain structure and cofactor dependency (Newton, 2001).

C1 domain of classical and novel PKCs represents a tandem repeat. Atypical PKCs possess only one copy of C1 domain termed atypical C1 that does not bind DAG and PE due to compromised face of the ligand-binding pocket. C2 domain is found in classical and novel PKCs. The C2 domain of cPKCs binds to the anionic lipids in the membrane in a Ca^{2+}-dependent manner, whereas novel C2 domain does not bind Ca^{2+} due to the absence of key aspartic acid residues involved in coordination of Ca^{2+}. The highly conserved among all ABC kinases (PKA, PKB and PKC) catalytic domain (CD) comprises ATP binding (C3) and substrate binding (C4) domains PKC phosphorylates substrates on Ser or Thr residues, typically in the context of basic sequence, with modest selectivity for specific residues at particular positions. Despite the large number of PKC isotypes, substrate promiscuity and the expression of multiple isoforms in the same cell, individual PKC isoforms seem to have unique function. Protein kinase C function is regulated by: (1) phosphorylation–based maturation (2) catalytic activation by cofactor binding and (3) targeting through interaction with PKC-binding proteins (Newton, 2001).

1.3.2. Maturation of PKC involves Ser/Thr phosphorylation

PKC maturation involves phosphorylation of three highly conserved residues in the catalytic domain (Figure 1.5.). The first and rate-limiting step is phosphoinositide-dependent kinase (PDK-1)- mediated phosphorylation of threonine residue at the activation loop (AL) (Dutil *et al*., 1998). Newly synthesized, immature to signal PKC species associate with the membrane compartment via weak interaction of pseudosubstrate, C1 and C2 domains in an 'open' conformation that exposes the AL for phosphorylation by PDK-1 (Figure 1.6). The negative charge at the AL site aligns correctly the residues for catalysis and unmasks the entrance to the substrate-binding cavity, thereby being absolutely required for the PKC maturation (Orr and Newton, 1994). The initial transphosphorylation triggers autophosphorylation of the turn motif (TM) located at the proline-rich turn of the kinase domain.

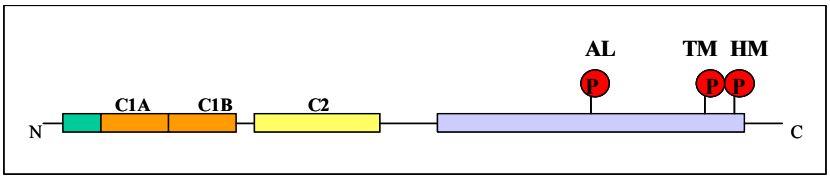


Figure 1.5. PKC maturation involves three ordered phosphorylation events. Shown are the conserved phosphorylation sites.

Whereas the AL phosphorylation is only necessary to initiate the autophosphorylation (Keranen *et al.*, 1995), the negative charge at the turn motif is necessary and sufficient for the function of mature PKC. PKC is locked in a catalytically competent, thermally stable and phosphatase resistant conformation via anchoring the C-terminus at the top of the upper lob of the kinase core. Following TM phosphorylation PKC rapidly autophosphorylates at the hydrophobic motif (HM)

by an intramolecular mechanism described for PKCβII (Behn-Krappa and Newton, 1999) or as shown for PKCδ by an upstream kinase possibly in complex with PKCζ (Ziegler *et al.*, 1999). HM phosphorylation is not essential for function but influences the subcellular localization and stability of PKC. Upon completion of these three ordered phosphorylations, catalytically competent, mature and resistant to phosphatases PKC is released into the cytosol, where it is maintained in an inactive conformation with the pseudosubstate sterically blocking the active site. Phosphatases counteract the phosphorylation state of PKC. Complete dephosphorylation is observed upon prolonged treatment with phorbol esters (Hansra *et al.*, 1999) and selective dephosphorylation of the HM of PKCε upon serum starvation (England *et al.*, 2001). Moreover, hydrophobic site transphosphorylation by PKCζ-complex requires active mTOR –mediated inhibition of phosphatases (Parekh *et al*., 2000).

1.3.3. PKC activation

Catalytically competent PKC could be activated by a myriad of stimuli (hormones, neurotransmitters, growth factors) that cause hydrolysis of membrane lipids. Conventional PKCs are activated downstream of receptor tyrosine kinases (RTK), nonreceptor tyroine kinases, as well as Gq-coupled receptors (GPCR) that activate phospholipase C (PLC)-mediated hydrolyses of PtdIns(4,5)P_2 to DAG and Ins(1,4,5)P_3. Ins(1,4,5)P_3 mobilizes Ca^{2+} release from intracellular stores. Elevated intracellular Ca^{2+} recruits PKC to the membrane via C2 domain and further C1 domain is engaged through DAG and PtdSer high-affinity binding (Figure 1.6.).

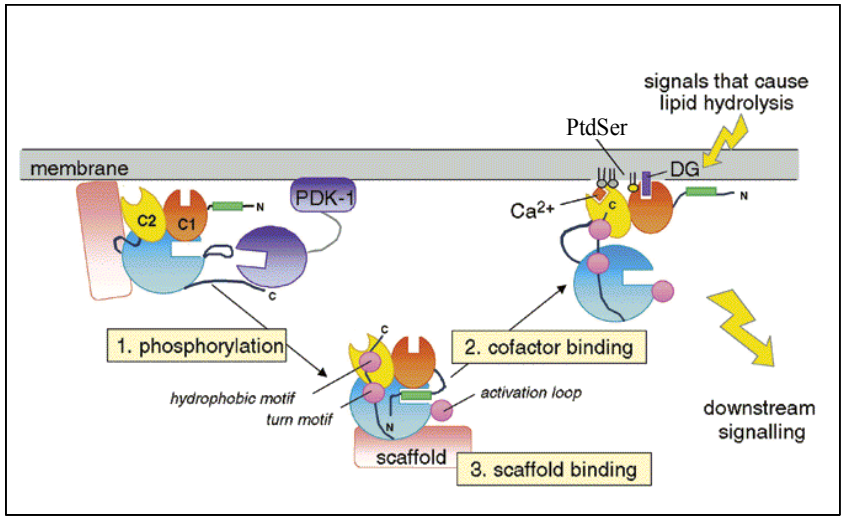


Figure 1.6. PKC life cycle from the biosynthesis to the catalytic activation (Newton, 2003).

Conformational switch induced by membrane targeting provides the energy for release of the pseudosubstrate from the active site. Thereby PKC adopts an active conformation allowing substrate binding and catalysis to proceed. The active state of PKC is highly sensitive to phosphatases and dephosphorylated PKC is targeted to proteolysis (ubiquitin-mediated or via

caveolin-dependent endosomal targeting). Binding of dephosphorylated TM to the molecular chaperone Hsp70 allows rephosphorylation and cycling back to the pool of functional PKC (Newton, 2003). This model of PKC life cycle and regulation, mainly valid for classical PKC isoforms, is more or less simplified. The regulation is much more complicated when we take into account that catalytic activation occurs by a variety of lipids and second messengers of lipid metabolism like phosphatidylcholine (PC)-derived DAG, saturated and unsaturated fatty acids (FA), ceramide, cholesterol sulfate, phosphatidic acid (PA), lysophosphatidylcholine (lysoPC), PIP_3 etc (Figure 1.7.). Lipid-protein interactions mediate translocation and subcellular redistribution in the process of PKC activation (Shirai and Saito, 2002).

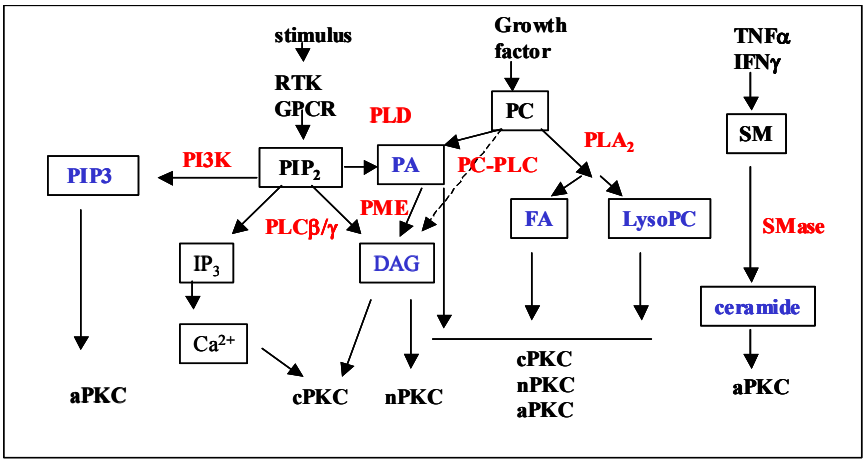


Figure 1.7. Lipids and products of the lipid metabolism that activate PKC. The PKC-activating lipids are labeled in blue, the lipid metabolizing enzymes in red. Based on Nishizuka, 1995

1.3.4. PKC-interacting proteins

Protein-protein interactions also target specific PKC isoforms to particular subcellular compartments (plasma membrane, nuclear membrane and perinuclear region, nucleus itself, Golgi complex) in a proximity to a distinct set of substrates and upstream activators providing PKC functional specificity (Jaken and Parker, 2000). PKCs directly interact with some of its substrates (STICKs, MARCKS, adducins, vinculin/talin), or with proteins that promote substarte phosphorylation (RACKs). PKC is mobile within the cell due to interaction with dynamic structures such as F-actin (PKCβ/ε) and caveolae (PKCα). Some PKC-interacting proteins bind determinants in the regulatory domain (tubulin binds PS of PKCζ, actin binding motif of PKCε is located between C1A and C1B, PH domain of Btk interacts with C1 domain, RACKs bind to C2/C2-like domain) while others bind to the kinase core (like PAR3/ASIP binds to V5 of PKCζ) (Ron D. and MG Kazanietz, 1999). Moreover, most of the interactions are isoform-specific (RACK/β'-COP2 and PKCε, LIP and PKCι/λ, p62/ZIP and aPKC). In addition to their scaffold function, some PKC binding proteins are able to influence PKC activity either in activating (LIP

and PKCλ) or inhibiting (PAR4 and aPKCs) manner. PKC activity is coupled to other signalling pathways through coordinated binding to scaffold proteins e.g. PKA, PKC, and phosphatase bound to AKAP79 (Klauck *et al*., 1996).

Among the PKC regulatory mechanism maturation seems to be the most conserved one, while both catalytic activation and targeting mechanisms are much more diverse and provide the basis for isoform-specific functions. PKC activity and function as well as its protein-protein interactions are subjected to control by covalent posttranslational modifications. In addition to the described above phosphorylation on serine/threonine residues, tyrosine residues are targeted by nitration and phosphorylation. Nitration of tyrosine residues appears to be a stable, even irreversible and selective modification altering protein activity and functions. For instance, nitric oxid-induced nitration of PKCε tyrosine residues is found to occur in the process of cardioprotection and promotes PKCε translocation and activation in the particulate fraction via enhanced PKCε –RACK2 interactions (Balafanova *et al*., 2002).

1.4. Tyrosine phosphorylation of PKC

1.4.1. Tyrosine phosphorylation in regulation of PKC activity and function

Recently, PKC modification by tyrosine phosphorylation has emerged as an additional mechanism that modulates its activity and function. In contrast to the serine/threonine phosphorylation, tyrosine phosphoryation patterns seem to be isoform-specific, rather than common for the whole PKC family. Tyrosine phosphorylation of several PKC isoforms (α, βI, βII, γ, δ, ε, θ, ζ and ι/λ) in different cellular context upon a variety of stimuli has been demonstrated (Table 1.) Most well investigated is the tyrosine phosphorylation of PKCδ (Kikkawa *et al*., 2002). Growth factor receptors (insulin receptor, PDGF receptor) or nonreceptor tyrosine kinases (Src, Fyn, Lyn, Lck, Abl, Pyk2) are implicated as proximate kinases and in some cases direct interaction between the respective tyrosine kinase and PKCδ has been documented. The consequence of PKCδ tyrosine phosphorylation seems to be cell type and stimulus-specific-inhibition of tyrosine phosphorylated PKCδ (Denning *et al*., 1993), change in the substrate specificity (Haleem-Smith *et al*., 1995) as well as catalytic activation (Konishi *et al*., 1997) or no influence being consequence but not prerequisite for catalytic activation (Ishikawa *et al*., 1996).

The tyrosine phosphorylation of PKC in many cases occurs coincident with its membrane translocation, but H_2O_2 induced-tyrosine phosphorylation and activation of PKC is not accompanied by membrane translocation. Tyrosine phosphorylation of PKCδ has been shown to target more than one tyrosine residue and different stimuli induce phosphorylation of different tyrosines. Distinct phosphorylation sites may regulate the PKCδ activity and biological functions

differently (Figure 1.8.). Furthermore, tyrosine phosphorylation of one and the same tyrosine residue may have different biological outcome in different cellular context. Tyrosine phosphorylation at Y311 in H_2O_2-treated COS-7 cells (Konishi *et al*., 2001) or by Lck *in vitro* (Konishi *et al*., 1997) activates PKCδ catalytic activity, while modification at the same residue by Src kinase (Blake *et al*., 1999) inactivates PKCδ and promotes its degradation. In various cell systems or in vitro assays tyrosine residues 52, 155 and 187 in the regulatory domain, tyrosines 311, 332 in the hinge region as well as tyrosines 512 and 565 in the catalytic domain of PKCδ have been reported as targets for phosphorylation.

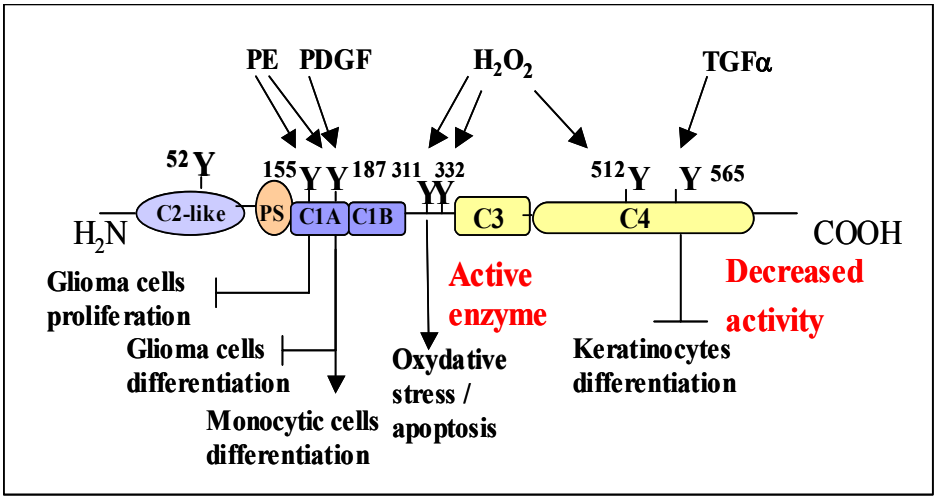


Figure 1.8. PKCδ tyrosine residues targeted by phosphorylation in response to various stimuli play different roles.

1.4.2. Tyrosine phosphorylation of atypical PKCs

Wooten *et al*. 2001a, have shown that the constitutive association of PKCι with the SH-3 domain of Src was enhanced by NGF in PC12 cells along with the formation of a signalling complex containing NGF receptor (TrkA), Src and PKC λ/ι. Moreover PKCι becomes tyrosine phosphorylated in the membrane in Src-dependent manner coincident with its activation by NGF. Tyrosines 256, 271 and 325 were identified as major sites phosphorylated by Src in the catalytic domain of PKCι with Y325 phosphorylation critically involved in Src-mediated PKCι activation and in NGF-promoted survival in serum-free media. This finding implicates receptor-induced and Src kinase-mediated tyrosine phosphorylation as a novel mechanism of activation of atypical PKC. NGF-induced tyrosine phosphorylation may also regulate the subcellular localization of atypical PKCs- phosphotyrosine 256 was shown to facilitate the nuclear import of atypical PKC (White *et al*., 2002) and tyrosine phopshorylation-dependent formation of a complex with p62/ZIP to route the activated atypical PKC to the endosomal compartment (Samuels *et al*., 2001). PKCζ is tyrosine phosphorylated in complex with active Src (Seibenhener *et al*., 1999) in

Table 1. Tyrosine phosphorylation of PKC isoforms

Cell line	PKC	Stimulus	Tyr kinase / Interaction	PTyr (pY)	Translocation	Tyrosine phosphorylation role	Biological function	Reference
RBL-2H3 mast cell	PKCδ	FcεRI activation	Lyn/ FcεRI	Y52, 155,565	Membrane	Substrate recognition modification	Mast cells degranulation	Haleem-Smith *et al*., 1995; Szallasi *et al*., 1995
	PKCα PKCβI	FcεRI activation	Syk	Y658 (PKCα) Y662 (PKCβI)		Grb-2 binding site generation	Ras/Erk pathway activation	Kawakami *et al*., 2003
	PKCθ	FcεRI activation	Src and Lyn		Membrane			Liu *et al*., 2001
COS-7 cells	PKCδ (α, βI, γ, ε, ζ)	H_2O_2	Lck- PKCδ *in vitro*	Y311, 332, 512 (PKCδ)	No	Increased PKC activity	Oxidative stress apoptosis	Konishi *et al*., 1997; 2001
HaCaT	PKCδ	UV				Increased PKC activity	Apoptosis induction	Fukunaga *et al*., 2001
HeLa	PKCδ	γ-IFN; Ceramide			Golgi	Increased PKC activity	γ-IFN →JNK →SMase	Kajimoto *et al*., 2001
B Ly	PKCδ	B cell receptor			Membrane	Increased PKC activity		Barbazuk and Gold, 1999
Parotide/ pancreatic acinar cells	PKCδ	Carbachol, Substance P	Src Pyk2/ PKCδ			Increased PKC activity	GPCR - promoted fluid secretion	Soltoff *et al*., 1995 Benes *et al*., 2000; Wrenn *et al*., 2001

Cell line	PKC	Stimulus	Tyr kinase / Interaction	PTyr (pY)	Translocation	Tyrosine phosphorylation role	Biological function	Reference
v-ras^{Ha} / primary keratinocytes	PKCδ	TGFα secretion-EGF Ca^{2+}	Src Family kinase	Y52, 155, 187 Y64 Y565	No significant	Decreased PKC activity	Neoplastic development / Turn off of differentiation	Denning *et al*., 1993, 1996, 2000; Joseloff *et al*., 2002
C6 glioma cells	PKCδ	PMA, PDGF	Src Fyn-via Y187	Y155 Y187			Inhibition proliferation/ Differentiation	Kronfeld *et al*., 2000
		etoposide	Src, IR, β-PDGFR *in vitro*	Y64, 187	Nuclear translocation	PKCδ cleavage	Apoptosis	Blass *et al*., 2002
32D NIH 3T3	PKCδ	TPA		Y187 Y155		PKCδ Y187-Pi no effect PKCδ Y155F - tumorigenic	Monocytic differentiation Proliferation control	Li *et al*., 1994, 1996; Deszo *et al*., 2001; Acs *et al*., 2000
Skeletal muscle cultures	βII,ζ δ	Insulin	PKCδ-Src-IR		Membr.	Increased PKC activity	Glucose uptake	Braiman *et al*.,1999; Rosenweig *et al*., 2004
	α, βII,δ	TPA						
T cells	PKCθ	TCR activation	Lck	Y90	Lipid rafts		T-cell activation	Liu *et al*.,2000
Platelets	PKCθ	Adhesion receptors	PKCθ-Btk				Platelet activation	Crosby and Poole, 2002
PC12	PKC ι, PKCζ	NGF	PKC ι-Src PKCζ-Src	Y 256, 271, 325 (PKC ι)		Increased PKC activity	Neurite differentiation	Wooten et *al*., 2001a; Seibenhener *et al*., 1999

response to insulin in primary skeletal muscle cultures (Braiman *et al*., 1999) and upon hydrogen peroxide treatment of COS-7 cells (Konishi *et al*., 1997).

1.4.3. Cross-talk between serine/threonine protein kinases and tyrosine kinases

In the context of cellular signalling network a complex bi-directional physical and functional interaction takes place between serine-threonine and tyrosine kinase signalling consisting in regulation of tyrosine kinases by PKC-dependent phosphorylation and vice versa regulation of PKC by phosphorylation and interaction with tyrosine kinases. For example in platelets both Bruton's tyrosine kinase (Btk) and PKCθ are activated upon stimulation of adhesion receptors GP Ib-V-IX and GP VI (Crosby and Poole, 2002). Btk is tyrosine phosphorylated downstream of Src family kinases as well as phosphorylated on threonine residues in a PKC-dependent manner. PKCθ in turn becomes tyrosine phosphorylated in a manner dependent upon Src family and Btk kinase activities. A cross talk between Btk and PKCθ in platelets has been proposed, since PKCθ positively modulates activity of Btk, which in turn feeds back negatively upon PKCθ. Controversely, in murine mast cells PKC- mediated phosphorylation downregulates Btk (Yao *et al*., 1994). Moreover, in other cell types activating tyrosine phosphorylation of PKC by Btk (Kawakami *et al*., 2000) or Src family kinases (Liu *et al*., 2000) have been reported. Recently investigated is reciprocal cross talk between PKCδ and Fyn (Crosby and Poole, 2003) and between PKCα and Syk /Src (Pula *et al*., 2004) in platelets.

1.5. Protein kinase C zeta (PKCζ)

1.5.1. Structure

The zeta isotype of protein kinase C belongs to the atypical PKC subfamily together with the PKCι (human) and λ (mouse) orthologs. PKCζ consists of regulatory moiety comprising PB1 (Phox and Bem1p) domain in V1, pseudosubstrate sequence (PS) and C1 domain, and kinase domain (Hirai and Chida, 2003).

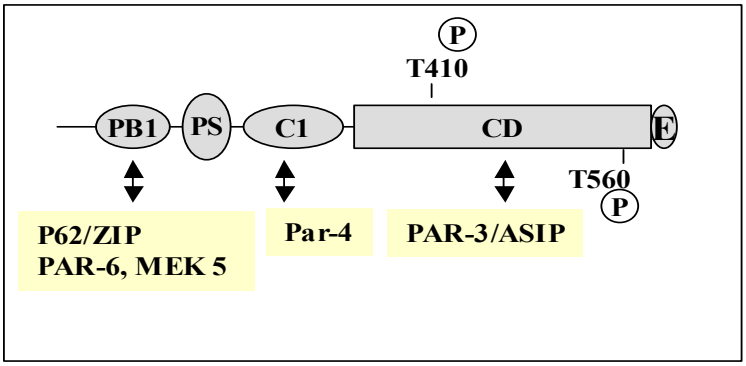


Figure 1.9. PKCζ domain structure. PKCζ consists of PB1, PS and C1 domains in the regulatory moiety and catalytic domain. Labeled are the AL and TM threonine residues, as well as the glutamate in the HM site. Shown are the main interacting partners for each of PKCζ domains.

Amino-terminally located is the unique among PKC isoforms PB1 domain involved in interactions with other PB1 motif- containing proteins, such as PAR-6, ZIP/p62 and MEK5 (Lamark *et al*., 2003; Wilson *et al*., 2003). PKCζ pocesses atypical C1 domain and does not respond to the second messenger DAG and PE. In addition PKCζ is Ca^{2+}-insensitive due to the lack of C2 domain. The activation loop and turn motif threonine residues in the cataylitic domain, respectively Thr-410 and Thr-560, are phosphorylated during maturation. The hydrophobic motif of atypical PKCs, in contrast to the others PKC isoforms, possesses a Glu residue instead of Ser/Thr that provides a negative charge (Figure 1.9.).

1.5.2. PKCζ activation mechanisms

PKCζ is activated by lipid components, such as phosphatidylinositols, phosphatidic acid, arachidonic acid and ceramide. PKCζ activation occurs downstream of PI3K in many signalling pathways- in monocytes, adipocytes, etc (Herrera-Velit et al., 1997; Standeart *et al*., 1997). PI3K product PIP3 contributes to PKCζ activation in both indirect and direct ways (Standaert *et al.*, 2001). The indirect modulation includes PIP3 binding to the PH domain of PDK1, and thereby activated PDK1-mediated phosphorylation of Thr-410 in the activation loop of PKCζ. This phosphorylation is essential for PKCζ activation- a T410A mutant loses enzymatic activity and respectively T410E mutant, probably mimicking the negative charge of the phosphorylated threonine, retains the activity. Phosphorylation by PDK1 triggers autophosphorylation of Thr560 in the turn motif. Although PKCζ has no PIP3-binding region, PIP3 implies a direct modulation on PS-dependent autoinhibition (Figure 1.10).

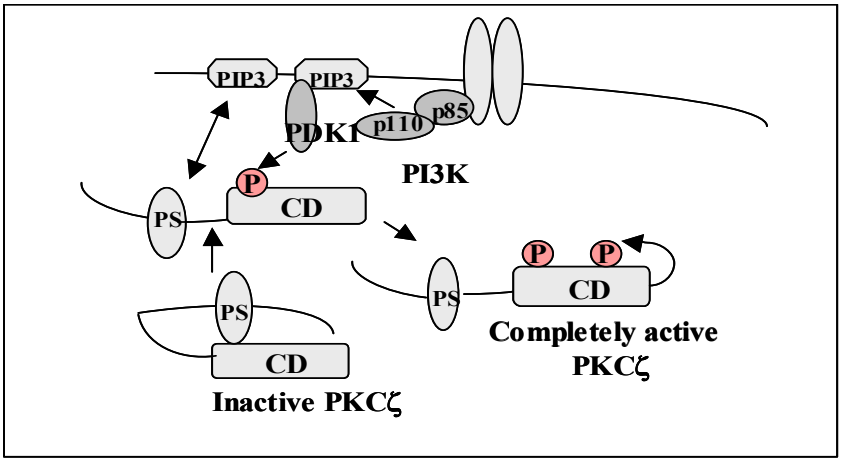


Figure 1.10. Activation of PKCζ downstream of PI3K and PDK1 activation via phosphorylation and PIP3 interaction. Adapted from Hirai and Chida, 2003.

Furthermore, PKCζ activity is modulated via specific protein-protein interactions (Figure 1.9.). Prostate apoptosis response-4 (Par-4) interacts with the C1 motifs of aPKCs and inhibits their activities. The overexpression of Par-4 causes cells to undergo apoptosis dependent on the block

of aPKC activity (Diaz-Meco *et al*., 1996). A product of *Caenorhabditis elegans* partitioning defective gene-3 (PAR-3) and its mammalian homolog ASIP (aPKC-specific interacting protein) bind the kinase domain of aPKCs and also inhibit its activity. PAR-6 binds to the PB1 domain of aPKCs. Furthermore, PAR-6, PAR-3 and aPKCs form a ternary complex, in which PAR-6 suppresses aPKC activity, and association of active Cdc42 releases the suppression (Lin *et al*., 2000) and association of nucleotide exchange factor ECT2 activates PKCζ (Liu *et al*., 2004). PKCζ binds to Ras (Diaz-Meco *et al*., 1994a). ZIP/p62 has been found to interact with atypical PKCs without modulation of PKCζ activity and being not a PKCζ substrate (Sanchez *et al*., 1998).

1.5.3. PKCζ role in MAPK cascade

PKCζ is involved in MAPK cascade activation in various cells in Raf-independent manner (Berra *et al*., 1995; Schoenwasser *et al*., 1998). Similarly, activated by lipopolysaccharide (LPS) PKCζ in human alveolar macrophages associates with MEK1 and activates MEK1, ERK1/2 but not Raf1 (Monick *et al*., 2000). It has been suggested that PKCζ functions as a MEK1 kinase independent of Raf1 pathway, but it is still unclear whether PKCζ phosphorylates MEK1 directly or indirectly. In HEK293 cells EGF-induced binding of endogenous PKCζ and MEK5 via PB1 domains is crucial for the increase in ERK5 activity (Diaz-Meco and Moscat, 2001). Function for PKCζ as adapter in ERK5 activation is proposed since the effect is independent of its activity. Similarly, PKCζ independently of its activity binds to and activates PKBγ/Akt3 (Hodgkinson *et al*., 2002).

1.5.4. PKCζ in receptor and other signalling complexes-the parallels

Atypical PKCs are example how to achieve functional specificity via protein adaptors (Moscat and Diaz-Meco, 2000). PKCζ is critically involved in the cell growth and survival signalling from TNFα, IL-1 and NGF receptors through the activation of NF-κB transcription factor. The interaction of PKCζ with those receptor-signalling complexes is mediated by PKC zeta-interacting protein (ZIP/p62) that harbors protein-protein interaction domains like PB1 and ZZ (zink finger). The mechanism of activation of PKCζ in the receptor complexes is not yet fully understood. ZIP/p62 links aPKCs to the TNFα receptor-signalling complex via selective interaction with the receptor-interacting protein RIP (Sanz *et al*., 1999) and to IL-1 (Sanz *et al*., 2000) and NGF receptor complexes via TRAF6 (Wooten *et al*., 2001b) (Figure 1.11.A). A scaffolding role for ZIP/p62 has been proposed, but in addition to its adaptor role ZIP/p62 may also regulate PKCζ activity since ZIP/p62 antagonizes Par-4 mediated PKCζ inhibition (Chang *et al*., 2002). The PB1 domain of PKCζ also interacts with PAR-6- another scaffold protein with

function in the control of cell polarity. PAR-6 interacts in addition with Cdc42 and Rac via CRIB-like domain and with PAR-3 via PDZ domain.

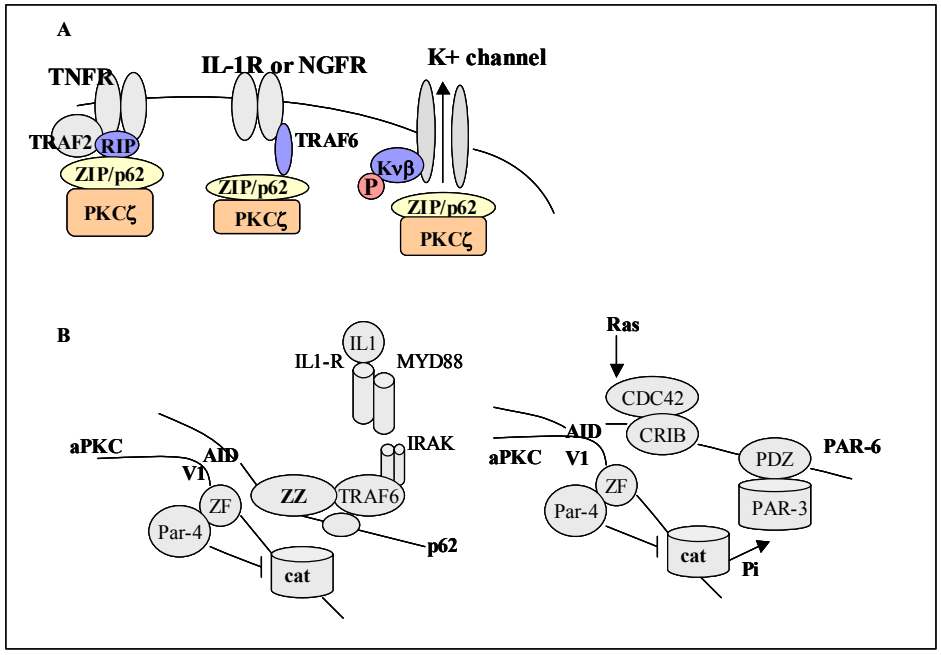


Figure 1.11. PKCζ in signalling complexes. (A). Recruited via ZIP/p62 of PKCζ to the receptor complexes (B). Parallel between the interaction of aPKC via p62 with IL1-R and via PAR-6 with Cdc42. V1 indicates the PB1 domain of aPKC. AID (aPKC-interacting domain) is motif within PB1 domains of p62 and PAR-6. ZF-zinc finger; cat-catalytic domain. Adapted from Hirai and Chida, 2003 and Moscat and Meco, 2000.

Mammalian PAR-3 (ASIP) is known to interact with the catalytic domain of aPKCs and to be relatively good substrate. There is an outstanding parallelism between ZIP/p62 and PAR-6: ZIP/p62 links in cytokine signalling PKCζ to the NF-κB pathway and PAR-6 seems to respond to Cdc42 signalling by linking the aPKCs with the actin cytoskeletal structure (Figure 1.11B). This provides mechanistic explanation for the requirement of the aPKCs in both Ras- and Cdc42-induced cell transformation (Qui *et al*., 2000). ZIP/p62 targets PKCζ activity to the auxiliary β subunit of the potassium channel (Gong *et al*., 1999) or to Grb14 (Cariou *et al*., 2002).

1.5.5. PKCζ-pro-apoptotic or pro-sirvival signalling

Protein kinase C zeta mediates anti-apoptotic, pro-survival signals, based on its ability to regulate ERK-AP1 and NF-κB pathways. PKCζ is involved as IκB kinase (IKK) kinase in the NF-κB pathway. IKK phosphorylates the inhibitor protein I κB and triggers its proteolitic degradation leading to the release of NF-κB allowing its nuclear translocation. Upon TNFα stimulation PKCζ and IKKβ interact through each catalytic domain thereby activating IKKβ (Lallena *et al*., 1999). TNFα-induced IKKβ activation respectively NF-κB pathway is impaired in PKCζ-deficient mice (Leitges *et al*., 2001). Frutos *et al*., 1999, have demonstrated that PKCζ contributes to the UV-induced apoptotic cell death via its caspase-3-mediated cleavage that generates inactive fragment corresponding to the catalytic domain. This is in keeping with the notion that a pro-survival enzyme like PKCζ needs to be blocked for apoptosis to proceed. Partly PKCζ inactivation during

apoptosis is mediated via Par-4 activation. PKCζ contributes to pro-survival signalling in many cases of drug-induced chemoresistance as a protective signal that is activated in tumor cells exposed to cytotoxic agents. Daunorubicin promotes pro-survival activation of Raf-1/MEK/ERK cascade with the critical upstream activation of PKCζ via PC-PLC-derived DAG and ROS-dependent PI-3K activation (Mansat-De Mas *et al*., 2003). In addition to signalling via MAPK pathway PKCζ can exert NF-κB-mediated protective effects against VP-16 (etoposide)-induced cytotoxicity (Filomenko *et al*., 2002). Thereby PKCζ represents an attractive target for chemosensitization of tumor cells. On the other hand, through Akt inhibition PKCζ provides anti-survival signals. In breast cancer cells PKCζ inhibit growth factor-induced activation of pro-survival PKB/Akt (Mao *et al*., 2000). PKCζ has been implicated in ceramide-induced Akt inhibition and growth arrest (Bourbon *et al*., 2002). The mechanism of ceramide-promoted cell death is related to PKCζ-dependent suppression of 3-phosphoinositide binding to the PH domain of PKB/Akt (Powell *et al*., 2003). Muller *et al*., 1995 suggested the role of PKCζ as bifunctional pro- and anti-survival modulator on the basis of different lipid cofactors.

1.5.6. Other cellular functions of PKCζ

PKCζ-deficient mice develop normally, but exhibit mildly impaired maturation of B cells and reduced number of Peyer's patches (Martin *et al*., 2002). PKCζ takes part in the PI3K-p70S6K signaling complex but is not sufficient for the complete activation of p70S6K (Romanelli *et al*., 1999). Insulin-stimulated via PI3K and PDK1 PKCζ has been directly implicated in the translocation of GLUT4 and glucose uptake in adipocytes (Bandyopadhyay *et al*., 1999). Via the ternary complex with PAR-3 and PAR-6 aPKCs controls cell polarity (Ohno *et al*., 2001) and the tight junction formation (Suzuki *et al*., 2001). Specifically in the brain a 51kDa protein referred to as PKMζ and representing the catalytic domain of PKCζ was detected and found to be both sufficient and necessary for maintenance of long-term potentiation (LTP) and memory (Ling *et al*.,2002).

1.6. AIM OF THE CURRENT WORK

The signalling pathways from GPCRs towards ERK/MAPK activation involving EGFR transactivation are relatively good investigated. Little is known about the EGFR-mediated regulation of the constituents of these signalling pathways via tyrosine phosphorylation. Recently, many lines of evidence demonstrate tyrosine phosphorylation of PKC isoforms upon stimulation of cell surface receptors and with critrical involvement of cytoplasmic tyrosine kinases. PKCs are main elements of both GPCR and RTK signalling implicated in their cross talk. However, little is known about the role of PKC tyrosine phosphorylation in the cross talk between GPCRs and RTKs.

The aim of the present work is to investigate the effect of EGFR stimulation on the tyrosine phosphorylation state of different PKC isoforms with the focus on the characterization of EGFR-mediated tyrosine phosphorylation of PKCζ. The involvement of EGFR and Src family tyrosine kinases as well as of PI3K in the PKC modification will be addressed. A potential physical association between EGFR and PKCζ will be investigated in detail. Another point of interest is to identify specific tyrosine residues targeted by EGFR-mediated phosphorylation. The effect of EGFR-mediated phosphorylation on PKCζ activity state will be analysed. We asked also the question whether the transactivated by GPCRs EGFR might also mediate tyrosine phosphorylation of PKCζ. In addition, the involvement of PKCζ tyrosine phosphorylation on EGFR downstream signalling events will be assessed.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Cell lines

COS-7 cell line – fibroblasts derived from the kidney of green African monkey (American Type Collection Culture-ATCC)
MCF-7 cell line - human breast adenocarcinoma (Deutsche Sammlung für Mikroorganismen und Zellkulturen-DSMZ)
A431 cell line - human epidermoid carcinoma (ATCC)

2.1.2. Chemicals and medium for cell culture, transfection, cell stimulation and lysis

Dulbecco's modified Eagle's medium (DMEM) high glucose with sodium pyruvate and L-glutamine, fetal calf serum (FCS), antibiotic-antimycotic mixture (10000 u/ml penicillin G, 10mg/ml streptomycin sulfate, 25 μg/ml amphotericin B), Gentamicin (10 mg/ml), Trypsin and Lipofectamine 2000 transfection reagent were from Invitrogen life technologies.
Other reagents were obtained as follow: Polyfect transfection reagent (Qiagen), human recombinant epidermal growth factor receptor (EGF), heparin-binding epidermal growth factor (HB-EGF), AG1478, LY294002, wortmannin, phorbol-12-myristate-13-acetate (PMA) (Calbiochem), hydrogen peroxide (Fluka), PMSF (Serva), diethylaminoethyl (DEAE)-dextran, chloroquine, sodium orthovanadate, aprotinin, leupeptine, oleoyl-L-α-lysophosphatidic acid (LPA), isoproterenol, cytosine β-D-arabinofuranoside (Ara-C) (Sigma).
Phosphate-buffered saline (PBS) used during transfections and for washes upon cell stimulation: 0.137M NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.7mM KH_2PO_4 pH 7.4

2.1.3. Bacterial strain and medium for bacterial cultivation

Escherichia coli **XL-1Blue** (Stratagene)

Luria-Bertani (LB) medium (Sambrook *et. al*., 1989) containing Bactotrypton – 10 g/l, yeast extract – 5 g/l, NaCl - 10 g/l, pH 7.0 supplemented with appropriate antibiotics- ampicillin 100 μg/ml or kanamycin 50 μg /ml and for plates solidified with 15 g/l agar.

2.1.4. Antibodies

Anti- **PKCα, βI, δ, ε, η, θ** and **ζ** isoform- rabbit polyclonal antibodies (Santa Cruz Biotechnology) (WB dilution 1:2000).

Anti-**EGFR** antibodies: mouse monoclonal 13G8 (Nanotools) (WB dilution 1:333); mouse monoclonal clone 425 (Merck) and mouse monoclonal (Santa Cruz Biotechnology) (IP).

Anti-**phosphotyrosine** antibodies: mouse monoclonal PY99 (Santa Cruz Biotechnology) and 4G10 (Upstate) (WB dilution: 1:3500-1:5000).

Anti-**phosphothreonine** mouse monoclonal antibody (Santa Cruz Biotechnology) (WB dilution: 1:100).

Anti-**phospho-p44/42 MAPK** (Thr 202/Tyr204) mouse monoclonal antibody (Cell Signaling) (WB dilution 1:2000).

Anti-**Erk1** and anti-**Erk2** rabbit polyclonal antibodies (Santa Cruz Biotechnology) (WB dilution 1:1500).

Anti-**phospho-Akt** (Ser473) rabbit polyclonal antibody (Cell Signaling) (WB dilution 1:1000).

Anti-**Akt** rabbit polyclonal antibody (BD Biosciences Pharmingen) (WB dilution 1:1000).

Anti-**HA.11 tag** mouse monoclonal antibody (Babco and Santa Cruz Biotechnology) (IP) (WB dilution 1:4000).

Anti-**T7 tag** mouse monoclonal antibody (Novagen) (WB dilution 1:4000).

Anti-**phospho- IκBα** (Ser32/36) mouse monoclonal antibody (Cell Signaling) (WB dilution 1:1000).

Anti **beta-actin** mouse monoclonal kindly provided by Dr. T. Bondeva (WB dilution 1:5000).

Anti-**PARP** rabbit polyclonal antibody (Roche) (WB dilution 1:2000).

Anti-**Src** mouse monoclonal clone 327 antibody (Oncogene) (WB dilution 1:1000).

Anti-**GFP** mouse monoclonal clone antibody (Santa Cruz Biotechnology) (WB dilution 1:500).

Secondary antibodies: horseradish peroxidase **(HRP)-conjugated anti-mouse and anti-rabbit** (Santa Cruz Biotechnology) (WB dilution from 1:2000 up to 1:10000).

Normal rabbit IgG – (Santa Cruz Biotechnology) (IP).

2.1.5. Plasmid vectors

PKC constructs: PKCα, **βI**, **δ**, **ε**, **η**, **θ** and **ζ**- wild type, human cDNAs in CMV promoter-driven expression vector (pcDNA3.0) (generous gift of Prof. K. Seedorf); **PKCζ constitutively active** (A to E mutation within the pseudosubstrate) and **PKCζ inactive mutant** (K281R) (described in Adomeit *et al*., 1999); **PKCζ-EYFP-N1, PKCζ-ECFP-N1, PKCζ-T410A-EYFP-N1 and PKCζ-T410A-ECFP-N1** (kindly provided by Dr. A. Hausser and Prof. K. Pfitzenmaier, Institute

of Cell Biology and Immunology, Stuttgart, Germany); **PKCζ-Y417F** (created by site-directed mutagenesis as described below).

EGFR constructs: EGFR-WT, EGFR-Y1173F, EGFR-Y992F, EGFR-Y1086F- in pRK5 (generously provided by Prof. F. Boehmer, FSU Jena, Jena, Germany); **EGFR-Y845F** in pcDNA3 (kindly provided by Prof. Dr. S. J. Parsons, University of Virginia, Virginia, USA); **EGFR-CFP-N1** (gift of Prof. Dr. L.E. Samelson, NIH, Bethesda, USA); **EGFR-ΔCR1 (Δ242-259)** dimerization mutant (provided by Prof. A.W. Burgess, Ludwig Institute for Cancer Research, Melbourne, Australia).

Other vectors: Human **HA-MAPK** in pcDNA3.0 was a kind gift of J. S. Gutkind (NIH, Bethesda, USA); mouse **SrcWT** and Src dominant negative mutant (**SrcRF-**K295R/Y527F) were from the lab of Joan Brugge (Harvard Medical School, USA); **PTEN-T7** in pCGT7 vector, was generous gift of Prof. F. Boehmer (FSU Jena, Germany), **p110betaWT** and **p110betaKR** constructs were generous gift of Prof. R. Wetzker (FSU Jena, Germany).

2.1.6. Other reagents

Enhanced chemiluminescence (ECL) detection reagent, [methyl-^{3}H]-Thymidine (2 Ci/mmol), protein A-Sepharose CL-4B, protein G-Sepharose 4 Fast Flow and Hybond polyvinylidene difluoride (PVDF) membrane were from Amersham Pharmacia Biotech.
[γ-^{32}P] adenosine triphosphate (ATP) (3000 Ci/mmol) was from NEN Life Science Products.
Other reagents and materials were as follow: myelin basic protein (MBP), BSA, ATP, Sepharose CL-4B, Tween 20, Triton X-100, glycerol 2-phosphate, sodium dodecyl sulfate (SDS), ethylene glycol-bis (β-aminoethylether)-N, N, N', N'-tertaacetic acid (EGTA) (Sigma), agarose broad range, Rotiphorese Gel 30 (37.5:1), ethylendiamine tetraacetic acid (EDTA), Glycin, Tris -(hydroxymethyl)-aminometan (Tris), NaCl, morpholinepropanesulfonic acid (MOPS) (Roth), Mowiol 4-88 reagent (Calbiochem), paraformaldehide (Merck), T-MAT Plus DG Film and Biomax MR Film (Kodak), prestained protein molecular weight marker (Fermentas).

2.2. Methods

2.2.1. Transfection methods

2.2.1.1. DEAE-dextran transient transfection

1.4-1.6 x 10^{6} COS-7 cells were seeded per 100mm dish in 10ml DMEM supplemented with 10% FCS and 15 μg/ml gentamicin. Cells were incubated at 37^{o}C and 5% CO_2. The next day

subconfluent cells were washed twice with 5 ml phosphate buffered saline (PBS) 0.137M NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.7mM KH_2PO_4 pH 7.4) and incubated with DNA mix in 600µl 0.67 mM DEAE-dextran in PBS. In order to achieve equal expression levels various amounts of DNA from different DNA constructs was used as indicated in the figure legends. In most of the cases 100 ng/10^5 cells PKC expression vectors were transfected along with 100 ng/10^5cells EGFR WT, Y992F, Y1086F, Y1173F constructs. The overall DNA amount upon co-transfection in most of the cases was not exceeding 200ng/10^5 cells DNA exept of the experiments that involve overexpression of EGFR-ΔCR1 (200 ng/10^5 cells to achieve the same expression as upon transfection of 20 ng/10^5 cells EGFR WT) and EGFR Y845F (6 to 10 fold more DNA than EGFR WT construct). The cells were incubated with the DNA mix for 30 min at 37^{o}C, then 6 ml serum-free DMEM supplemented with 0.13 mM chloroquine were added and the cells were incubated for additional 3 h at 37^{o}C. Medium was exchanged with DMEM supplemented with 10% FCS and 1% antibiotic mixture and cells were grown for additional 48h. The cells were serum-starvated over night prior stimulation (as described in fugure legends) and lysed in lysis buffer (see below in 2.2.2.). In most of the experiments transient transfection was performed by DEAE-dextran method, if not otherwise indicated.

2.2.1.2. PolyFect transfection

The day before transfection 5 x 10^4 COS-7 cells were seeded per well in 24-well plate in 1 ml DMEM supplemented with 10% FCS and 15 µg/ml gentamicin. The next day subconfluent cells were washed twice with 1 ml PBS and 750 µl DMEM with 10% FCS and 1% antibiotics was added per well. Separately, 180 ng DNA per transfection was diluted with serum-free medium up to 15µl and 1.5µl of PolyFect transfection reagent was added, mixed by pipetting and the samples were incubated for 10 min at room temperature to allow complex formation. Then the transfection complexes were transferred to the cells diluted up to 250 µl/well DMEM with 10% FCS and 1% antibiotics. The cells were incubated with the complexes for 48 h and then proceeded with [^{3}H]-Td incorporation for proliferation analyses.

2.2.1.3. Lipofectamine 2000 transfection

The day before transfection 2.4 x 10^5 COS-7 cells were seeded per well in 6-well plate in 2 ml DMEM supplemented with 10% FCS without antibiotics. The next day the medium was changed to serum-free medium. For each transfection 4 µg DNA was diluted up to 250 µl with serum-free medium. Separately 10 µl Lipofectamine 2000 was diluted up to 250 µl with serum-free medium, incubated at room temperature and mixed with the diluted DNA within 5 min. The mixtures were

incubated for 20 min at room temperature to allow formation of the DNA-Lipofectamine 2000 complexes and were added to the cells. After 5 h incubation with the complexes medium was changed to DMEM supplemented with 10% FCS without antibiotics and the cells were grown for 48 h before further analysis.

2.2.2. Preparation of cell lysates

Before lysis the cells were pretreated with appropriate inhibitors for 30 min at 37^{o}C and stimulated as indicated in the figure legends, washed in ice-cold PBS and lysed on ice in a lysis buffer (LB) containing 20 mM Hepes, pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 1% Triton X-100, 2.5 mM $MgCl_2$, 1mM dithiothreitol (DTT), 2mM sodium vanadate, 1mM phenylmethylsulfonylfluoride (PMSF), 20 μg/ml aprotinin and 20 μg/ml leupeptin. Total cell lysates were centrifuged at 14,000xg for 5 min at 4^{o}C to pellet the nuclei and the supernatants were stored at -80^{o}C.

2.2.3. Preparation of cell lysates for PARP

Extraction buffer was prepared by mixing per each sample 100 μl of 50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1 mM PMSF and 50 μl of 50 mM Tris-HCl, pH 6.8, 6 M urea, 6% β-mercaptoethanol, 3% SDS and 0.003% bromphenolblue. Whole cell lysates were sonicated (one 60 s pulse at 180V) and incubated for 15 min at 65^{o}C.

2.2.4. Colocalization of PKC zeta and EGFR

Subconfluent COS-7 cells grown in 6 well plates on glass cover slips were transfected with PKCζ- EYFP and EGFR-CFP using Lipofectamine 2000. Serum-starved cells were stimulated with 10 nM EGF for 1 min, washed in PBS (0.1 M NaCl, 0.063 M Na_2HPO_4, 0.022 M NaH_2PO_4 pH 7.4), fixed with 3.5% paraformaldehide in PBS for 20 min at room temperature and after extensive wash with PBS and final wash with bidestiled water the cover slips were mounted on glass slides with Mowiol. Mowiol was prepared by stirring 2.4g in 6g glycerol and 6 ml destilled water for 2 h at room temperature, followed by addition of 12 ml 0.2M Tris-HCl, pH 8.5 and incubation at 50-60^{o}C for 10 min. Aliquates were stored at –20^{o}C. The slides were kept in dark until detection of sub-cellular localization of fluorescent proteins by laser scanning microscopy. Fluorescent specimen were analysed with Leica SP2 confocal microscope with argon laser and imaged using the Leica TCS software.

2.2.5. SDS-PAGE and Western blotting

SDS-PAGE gels (7.5-13%) gels were used as follow: 10% for PKC detection, 7,5%-9% for EGFR detection, 8-9% for co-immunoprecipitations of EGFR and PKC, 13% for MBP phosphorylation, 12,5% for phospho-Erk and phospho-Akt detection. Cell lysates corresponding to 10^5 cells boiled in 1x Leammli buffer for 5 min and clarified by centrifugation for 2 min at 14000 rpm were run per lane with constant current 22 mA/gel and maximal voltage 150V.

Resolving gel: 7.5-13% PA, 380 mM Tris-HCl, pH 8.8, 0.1% SDS

Stacking gel: 4% PA, 125 mM Tris-HCl, pH 6.8, 0.1% SDS

Running buffer: 25 mM Tris, 200mM glycin, 0.1% SDS

Laemmli sample buffer (3x): 6% SDS, 4.32 M β-mercaptoethanol, 40% glycerol and bromphenol blue.

Resolved proteins were electrotransferred to Hybond PVDF membranes using semi-dry blotting with constant current 0.8 mA per cm^2 membrane and maximal voltage 12V.

Anode buffer: 0.3M Tris, 20% methanol.

Cathode buffer: 25mM Tris, 40mM 6-aminohexanoic acid, 20% methanol and 0.05% SDS (freshly added).

Background blocking was performed by incubating the membranes with 1% BSA and 1% dry milk (polyclonal antibodies), 1% BSA (for monoclonal antibodies), 5% dry milk (for phospho-Erk, phospho-IκBα and Erk2-specific antibodies), 5% BSA (for anti-phospho-Akt antibody). Blocking solutions were prepared with **Tris-buffered saline** (TBS- 10 mM Tris, pH 7.5, 150 mM NaCl) for overnight blocking and in TBS with 0.1% Tween-20 for 1 hour blocking at room temperature. Membranes were incubated with the most of the primary antibodies for 1 h at room temperature (with phospho-Erk, Akt and IκBα-specific antibodies incubation was carried out over night at 4^{o}C). After triple wash with TBS supplemented with 0.1% Tween-20 membranes were incubated with the respective secondary antibodies: anti-mouse or anti-rabbit HRP- conjugated diluted 1:3000 up to 1:10000. After four times wash with TBS-0.1% Tween-20 the visualization was performed by ECL.
When necessary to reprobe with another antibody, the membrane was washed after ECL twice for 10 min in TBS-0.1% Tween-20 and stripped in 50 mM Tris-HCl, pH 6.8, 2% SDS and 11.5 mM

β-mercaptoethanol for 30 min at 55-70^{o}C. After extensive wash with TBS-0.1% Tween-20 membrane were reprobed with appropriate antibodies as described above.

2.2.6. Immunoprecipitation

Clarified cell lysates from 1.4x10^{6} COS-7 cells were incubated with 1-1.5 μg anti-PKCζ antibody or 1,5-2 μg anti-EGFR antibody clone 425 for 3 hours at 4^{o}C with rotation. The PKCζ antibody was substituted with normal rabbit IgG in the negative controls. The immunocomplexes were collected on Protein A-Sepharose (3 mg beads per sample) for additional 1,5 h at 4^{o}C with rotation. The beads with bound immuno-complexes were washed three times in phosphate-buffered saline PBS (0.1 M NaCl, 0.063 M Na_2HPO_4, 0.022 M NaH_2PO_4 pH 7.4) supplemented with 1% TritonX-100 and 2 mM sodium orthovanadate, boiled in Leammli buffer for 5 min and the supernatants were subjected to Western blotting using anti-phosphotyrosine antibody to detect the tyrosine phosphorylation, or PKCζ immunoprecipitates were probed with anti-EGFR antibody and EGFR immunoprecipitates with PKCζ antibody (to assess the co-immunoprecipitation), and after stripping reprobed anti-PKCζ antibody and anti-EGFR antibody 13G8, respectively (to verify equal loading of the immunoprecipitates). Equivalent expression levels of the DNA constructs were detected by Western blotting with appropriate antibodies.

2.2.7. HA-MAPK kinase activity assay

Cells were transfected with HA-MAPK along with PKCζ and EGFR expressing constructs by DEAE-dextran method. Clarified cell lysates were immunoprecipitated with anti-HA monoclonal antibody 12CA5 (Babco, Berkley, CA) for 1 hour at 4^{o}C and immunocomplexes were recovered on Protein G-sepharose beads for 30 min at 4^{o}C. The beads were washed three times with PBS supplemented with 1% TX-100 and 2 mM sodium orthovanadate, once with 0.5 M LiCl in 100 mM Tris-HCl (pH 7.5) and once with kinase reaction buffer (12.5 mM morpholinepropanesulfonic acid [pH 7.5], 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM sodium fluoride, 0.5 mM sodium vanadate). Reactions were performed in 30 μl of kinase buffer containing 1 μCi of [γ-^{32}P] ATP, 20 μM cold ATP, 3.3 μM DTT and 40 μg of myelin basic protein (MBP) at 30^{o}C for 20 min. Reactions were stopped by addition of Laemmli buffer, boiled for 5-10 min and separated on 13% SDS-polyacrylamide gels. Lower parts of the gels, containing phosphorylated MBP, were fixed in (45% methanol and 10% acetic acid), dried and MBP phoshorylation was visualized by autoradiography. The upper parts of the gels were electrotransferred on membranes and analysed with anti-HA antibody for immunoprecipitated of HA-MAPK.

2.2.8. PKC zeta kinase activity assay

The kinase activity of PKCζ was determined on PKCζ immunoprecipitates using myelin basic protein (MBP) as a substrate. PKCζ immunoprecipitation was carried out as for tyrosine phosphorylation and co-immunoprecipitation analysis. The sepharose beads with attached immune complexes were washed three times with complete lysis buffer (LB) followed by one wash in kinase buffer containing 20 mM Tris, pH 7.4, 50 mM NaCl, 7.5 mM $MgCl_2$, 0.5 mM EGTA and 2 mM sodium vanadate. Kinase reactions were carried out in 50 µl kinase buffer supplemented with 1 µCi of [γ-^{32}P] ATP, 20 µM cold ATP and 40 µg MBP for 20 min at 37^{o}C. Estimation of MBP phosphorylation was performed in the same manner as for MAPK activity. The upper parts of the gels were electrotransferred on membranes and analysed with phosphotyrosine-specific antibody or PKCζ-specific antibody and autophosphorylation of PKCζ was visulaized by autoradiography.

2.2.9. Site-directed mutagenesis

Y417F mutant of PKCζ was generated using QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's protocol based on the pcDNA3 construct containing wild type human PKCζ as a template. A pair of 35-mers fully complementary to each other primers was designed to contain the mutation – Y417Ff (sense strand, 5'-ctgcggaaccccgaatttcatcgcccccgaaatcc-3') and Y417Fr (antisense strand, 5'-ggatttcgggggcgatgaaattcggggttccgcag-3'). The whole plasmid containing 1779 bp coding for human PKCζ was amplified using PfuTurbo DNA polymerase. The PCR program included an initial denaturation step at 95^{o}C for 3 min followed by 16 cycles each consisting of denaturation step at 95^{o}C for 30 sec, annealing step at 54^{o}C for 1 min and polymerization step at 68^{o}C for 15 min (2 min/kb DNA). Template DNA was digested with DpnI restriction endonuclease for 3 h at 37^{o}C and PCR product was used for transformation of XL-1 Blue cells. The colonies were picked and plasmid DNA was isolated and sequenced for confirmation of the tyrosine (Y) 417 mutation to phenylalanine (F). Additional overlapping sequencing runs were performed in order to check the identity of the remaining PKCζ coding sequence.

2.2.10. Transformation of E.coli and plasmid DNA preparation

For competent cells preparation 1 ml overnight culture of XL-1 Blue was inoculated in 100 ml LB medium without antibiotics and incubated at 37^{o}C on shaker (200rpm) for 2-4 h until the culture reaches OD_{600} 0.3-0.6. The cells were pelleted by centrifugation for 5 min at 5000rpm, resuspended in 10 ml TSB (10% polyethylenglycol 6000, 5% dimethylsulfoxide (DMSO), 20 mM Mg^{2+} -10 mM $MgCl_2$ and 10 mM $MnCl_2$ in LB medium) and aliquots were kept at –80^{o}C.

E.coli competent cells (100 µl per sample) were thawed on ice and added to the 10-50 ng DNA in 1x KCM buffer (0.1 M KCl, 0.03 M $CaCl_2$, 0.05 M $MgCl_2$). Reaction was mixed and incubated on ice for 30 min, then transferred for 10 min at room temperature, followed by addition of 1ml LB without antibiotics and incubation for 1 h at 37^{o}C. For plasmid amplification 10 µl (or half of the transformation mixtures for the mutagenesis) were spread on plates with LB medium supplemented with appropriate antibiotics. The plates were incubated at 37^{o}C overnight. For plasmid DNA isolation single colonies were picked up and bacteria were grown in overnight culture (3 ml for miniprep extraction and 100 ml for maxiprep extraction of plasmid DNA) at 37°C by shaking (200 rpm). The cells were harvested by centrifugation and plasmid DNA was extracted using Qiagen Plasmid Isolation kit following the supplier's protocol.

Electrophoresis of plasmid DNA was carried out in 1% agarose gels with ethidium bromide (final concentration 2,5 µg/ml) in 1xTAE buffer (40 mM Tris, Acetic acid, 1.14 ml/l and EGTA- 0.03 g/l, pH 8) in horizontal apparatus at 80 V. DNA samples were resuspended in loading buffer (10x 30% glycerol, 1xTAE 0,025% bromophenol blue, 0,025% xylene-glycol). The size of DNA was estimated using 1 kb Ladder (BioLabs).

2.2.11. [3H]-thymidine incorporation assay

COS-7 cells were transfected in 24-well plates and 48 h later were serum starved for 24 h. The cells were stimulated with 10 nM EGF for 20 h, followed by addition of 1 µCi per well [^{3}H]-Td for additional 4 h. Cells were washed with ice-cold PBS, incubated with 10% TCA trichloracetic acid supplemented with 1% pyrophosphate (20 mM) for 30 min on ice and then subsequently washed twice with 1% TCA, 1% pyrophosphate, twice with 96% ethanol, followed by solubilization with 1 N NaOH for 10 min on ice with gentle shaking and neutralization with 2 N HCl. The samples were transferred into scintillation vials and the incorporation of [^{3}H]-Td into newly synthesized DNA was measured in Lumasafe Plus by Beckman scintillation counter.

3. RESULTS

3.1. Tyrosine phosphorylation of PKC isoforms overexpressed in COS-7 cells upon EGFR stimulation

In order to analyse the tyrosine phosphorylation of protein kinase C (PKC) induced by EGFR stimulation we transiently transfected COS-7 cells with pcDNA3.0 expression vectors of particular PKC isoforms (classical PKC isoforms α and βI, novel PKC δ, ε, η and θ and atypical PKC ζ). COS-7 cells express endogenously PKCα, βI, δ, ε and ζ (predominantly PKCα, βI and ζ) whereas Western blot analysis for PKCη and θ did not show detectable amount of endogenously expressed protein. Transient transfection led to significant overexpression of all PKC isoforms. The endogenous EGFR of COS-7 cell was stimulated with 100ng/ml (16.7nM) epidermal growth factor (EGF). First we tried to identify PKC tyrosine phosphorylation by Western blot of cell lysates with phosphotyrosine-specific antibody.

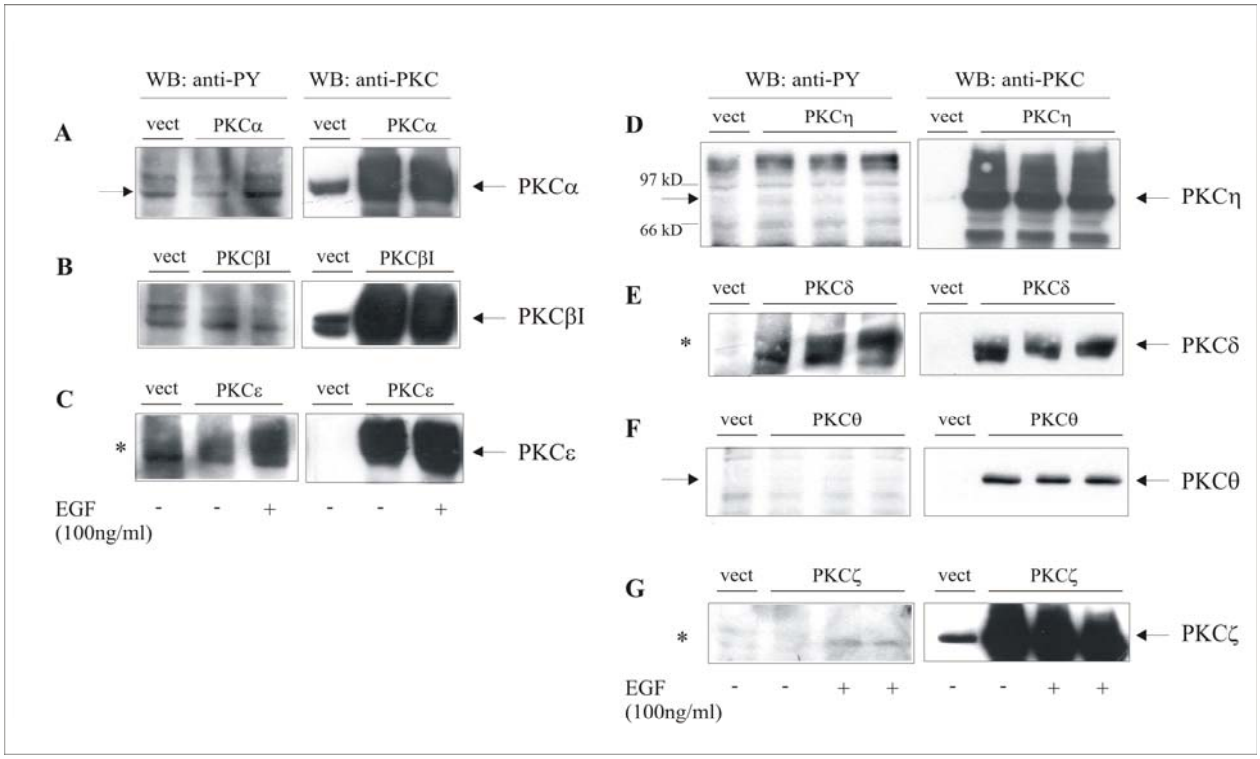


Figure 3.1. EGF-induced tyrosine phosphorylation of PKC isoforms overexpressed in COS-7 cells. Cells were transiently transfected with 150ng DNA/10^5 cells of either empty vector or expression constructs for PKCα (A), PKCβI (B), PKCε (C), PKCη (D), PKCδ (E), PKCθ (F) and PKCζ (G). Serum starved cells were left untreated or stimulated with 100ng/ml EGF for 5 min at 37°C. Cell lysates from 10^5 cells were resolved by 10% SDS-PAGE, electroblotted on the PVDF membrane and the membranes were probed with phosphotyrosine-specific antibody PY99. After stripping, the membranes were reprobed with PKC isoform-specific antibodies. Respective PKC isoforms are indicated by arrows. The increase in PKC tyrosine phosphorylation in response to EGF is labeled by star. Shown is a representative of four independent experiments.

On Figure 3.1 is shown that with this approach it was difficult to detect pronounced tyrosine phosphorylation of most of the overexpressed PKC isoforms. Only a weak phosphotyrosine signal was found corresponding to the positions of PKCη, θ and ζ and stronger tyrosine phosphorylated band was detected on the respective positions of PKCα, βI and ε and overexpressed PKCδ. This signal was not influenced either by PKC overexpression or upon EGF stimulation in the case of PKCβI and η. Increase in the tyrosine phosphorylation upon stimulation with 100ng/ml EGF was visible on Western blot only very weakly in the case of PKCθ, faint for PKCα and ζ as well as more pronounced increase in the case of PKCε and PKCδ. Tyrosine phosphorylated PKCε (C) and δ (E) showed reduced electrophoretic mobility. Since hydrogen peroxide and phorbol ester (PMA) are well known as PKC tyrosine phosphorylation inducers (Table 1), we compared their ability to induce tyrosine phosphorylation of PKCε and PKCδ with that of EGF. As it is demonstrated on Figure 3.2 the stimulation with 5mM hydrogen peroxide for 10 min led to high increase in the tyrosine phosphorylation of both PKCε and PKCδ. Both isoforms had similar patterns of tyrosine phosphorylation upon treatment with 100 nM PMA for 10 min but PMA effect was less pronounced compared to the tyrosine phosphorylation induced by hydrogen peroxide. Stimulation with 10 and 100ng/ml EGF induced also tyrosine phosphorylation of both PKC isoforms. The lower tyrosine phosporylation of PKCε induced by 100ng/ml EGF compared to 10ng/ml EGF stimulation can be explained by lower protein loading in that case. EGF treatment seems to be less potent in inducing PKC tyrosine phosphorylation than hydrogen peroxide but more effective than phorbol ester treatment.

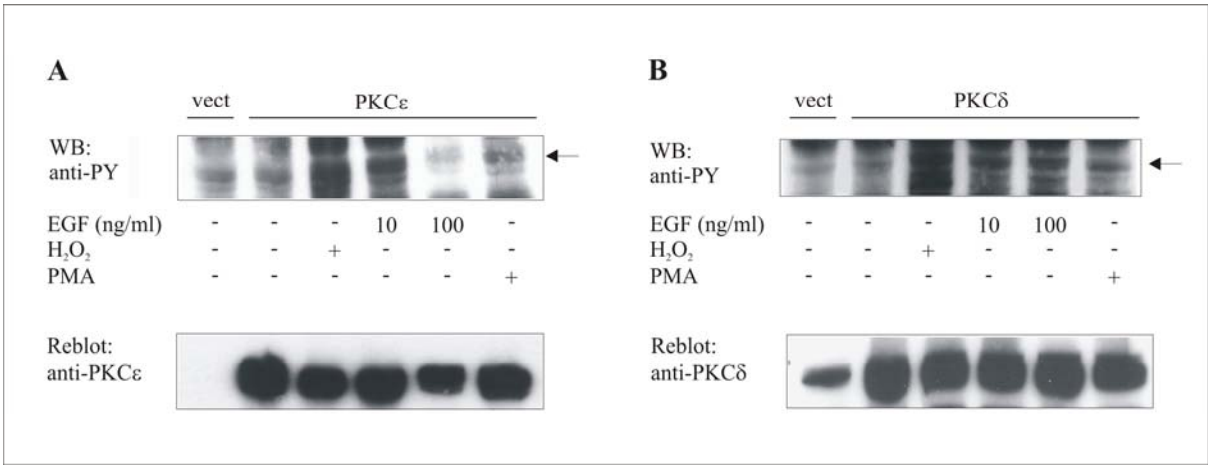


Figure 3.2. Tyrosine phosphorylation of PKCε and δ induced by EGF, H_2O_2 and PMA treatment in COS-7 cells. Cells transfected with PKCε (A), PKCδ (B) expression constructs or empty vector were serum starved and either left untreated or treated with 5mM H_2O_2 for 10 min, 10ng/ml EGF for 5 min, 100ng/ml EGF for 5 min, or 100nM PMA for 10 min as indicated. Protein extracts were analysed by Western blotting with phosphotyrosine-specific antibody PY99 and reprobed with PKC ε (A) or PKCδ (B)-specific antibodies, respectively. The arrows indicate the position of PKC on phosphotyrosine blot. Similar results were obtained in two additional experiments.

In this way we confirmed the already described EGF-induced tyrosine phosphorylation of PKCδ (Denning et al., 1993) and obtained some indications about tyrosine phosphorylation of other PKC isoforms as well. Despite of that, Western blot of cell lysates seemed to be not suitable for more detailed characterization of the PKC modification in response to EGF. Using phosphotyrosine blot of PKC immunoprecipitates we confirmed the EGF-induced tyrosine phosphorylation of PKCε and PKCζ observed on cell lysates (Figure 3.3.), whereas the results for PKCα were not conclusive and tyrosine phosphorylation of PKCβI was excluded (data not shown). Figure 3.3.A demonstrates that overexpressed PKCε is tyrosine phosphorylated upon short (2 to 3 min) stimulation with 10nM EGF. The tyrosine phosphorylation of the overexpressed PKCζ upon stimulation of the endogenous EGFR was more pronounced compared to that of PKCε (Figure 3.3.B).

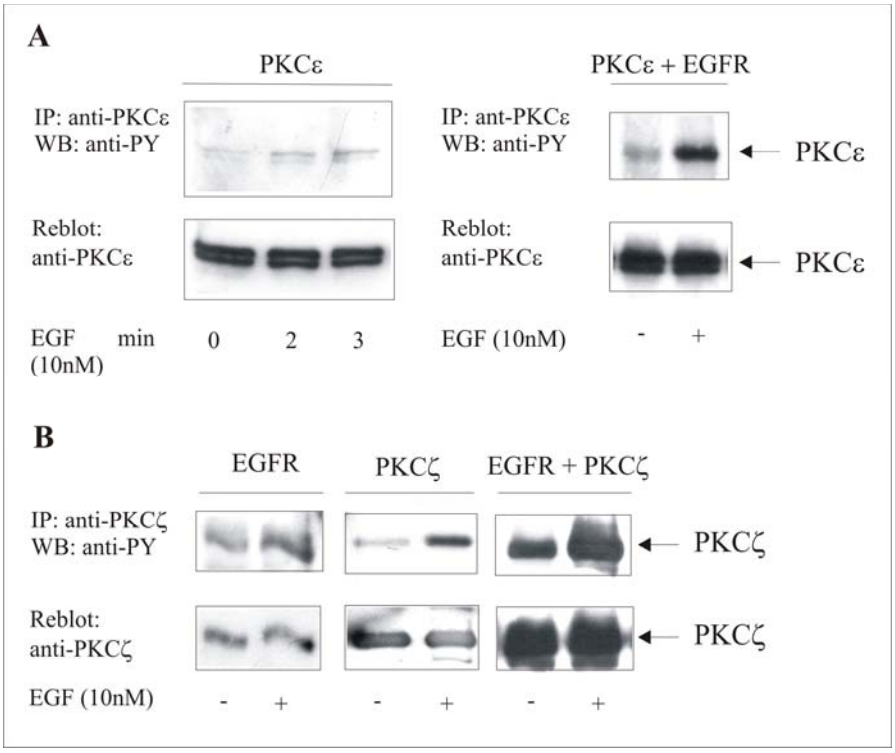


Figure 3.3. Tyrosine phosphorylation of PKCε and PKCζ upon EGF stimulation - detection in PKC immunoprecipitates. COS-7 cells were transfected with PKCε alone or together with EGFR coding vector (A), or EGFR alone, PKCζ alone and PKCζ in combination with EGFR (B). The amount of plasmid DNA used for transfection was 180 ng/10^5 cells for single transfection of PKCε, 100ng/10^5 cells for single transfections of EGFR and PKC ζ, and not more that 200ng/10^5 cells (125ng/10^5 cells PKC and 62.5ng/10^5 cells EGFR) in the case of double overexpression (2μg PKC and 1μg EGFR per 1.6x10^6 cells). PKCs were immunoprecipitated with isoform-specific antibodies from cell lysates of either untreated or EGF-stimulated COS-7 cells. EGF stimulation was for 5 min if not otherwise indicated. Immunoprecipitates were analysed by Western blotting with PY99 phosphotyrosine-specific antibody and reprobed with PKC isoform-specific antibodies. Similar results were obtained in three additional experiments.

Moreover, tyrosine phosphorylation of endogenous PKCζ upon stimulation of the overexpressed EGFR was detected as well. Tyrosine phosphorylation of endogenous PKCε upon stimulation of the overexpressed EGFR was not observed, probably due to the lower endogenous level of PKCε in COS-7 cells relatively to PKCζ (data not shown). However the phosphotyrosine signal for both PKC isoforms was not high enough to enable us to study quantitatively their tyrosine phosphorylation. Therefore we used a double overexpression system. COS-7 cells that overexpress transiently either PKC ζ or PKCε along with the EGFR showed pronounced increase in the basal and EGF-induced tyrosine phosphorylation of these PKC isoforms (Figure 3.3.).

As a result of these preliminary experiments we confirmed that EGF induces tyrosine phosphorylation of PKCδ and identified that two additional PKC isoforms are subjected to tyrosine phosphorylation upon EGFR stimulation in COS-7 cells- PKCζ and PKCε. Since the tyrosine phosphorylation of PKCδ is studied in detail (Denning *et al*., 1993, 1996, 2000) we focused our study on EGFR-induced tyrosine phosphorylation of PKCζ and PKCε.

3.2. EGF-induced tyrosine phosphorylation of PKC zeta and PKC epsilon

3.2.1. Stimulus concentration dependence and time course of PKC zeta and PKC epsilon tyrosine phosphorylation

Next we analysed EGFR-induced modification of PKCζ and PKCε by tyrosine phosphorylation with respect of the time course and EGF concentration dependence. On Figure 3.4 is compared the tyrosine phosphorylation of PKCζ and PKCε in response to EGF stimulation of endogenous EGFR (A and C) and co-overexpressed EGFR (B and D). EGFR-mediated tyrosine phosphorylation of both isoforms was dependent on the EGF concentration used for stimulation and substantially increased in the concentration range from 0.1nM up to 10nM EGF. The tyrosine phosphorylation observed upon endogenous EGFR stimulation is significantly less pronounced compared to the modification induced by treatment with 5 mM hydrogen peroxide for 10min as a positive control. The difference between both effects is extremely big in the case of PKCε (Figure 3.4.C). The concentration dependence in the case of overexpressed EGFR is shifted compared to the endogenous system with substantial increase in the tyrosine phosphorylation of PKCζ and PKCε upon stimulation with 1-10nM EGF. Comparable significant effect on the tyrosine phosphorylation of PKCζ under endogenous EGFR conditions is found only upon stimulation with high EGF concentration of 10nM and has not been observed for PKCε. Stimulation with 10nM EGF of COS-7 cells co-overexpressing EGFR along with either PKCζ or PKCε corresponds to the hydrogen peroxide treatment in its potency for inducing tyrosine phosphorylation.

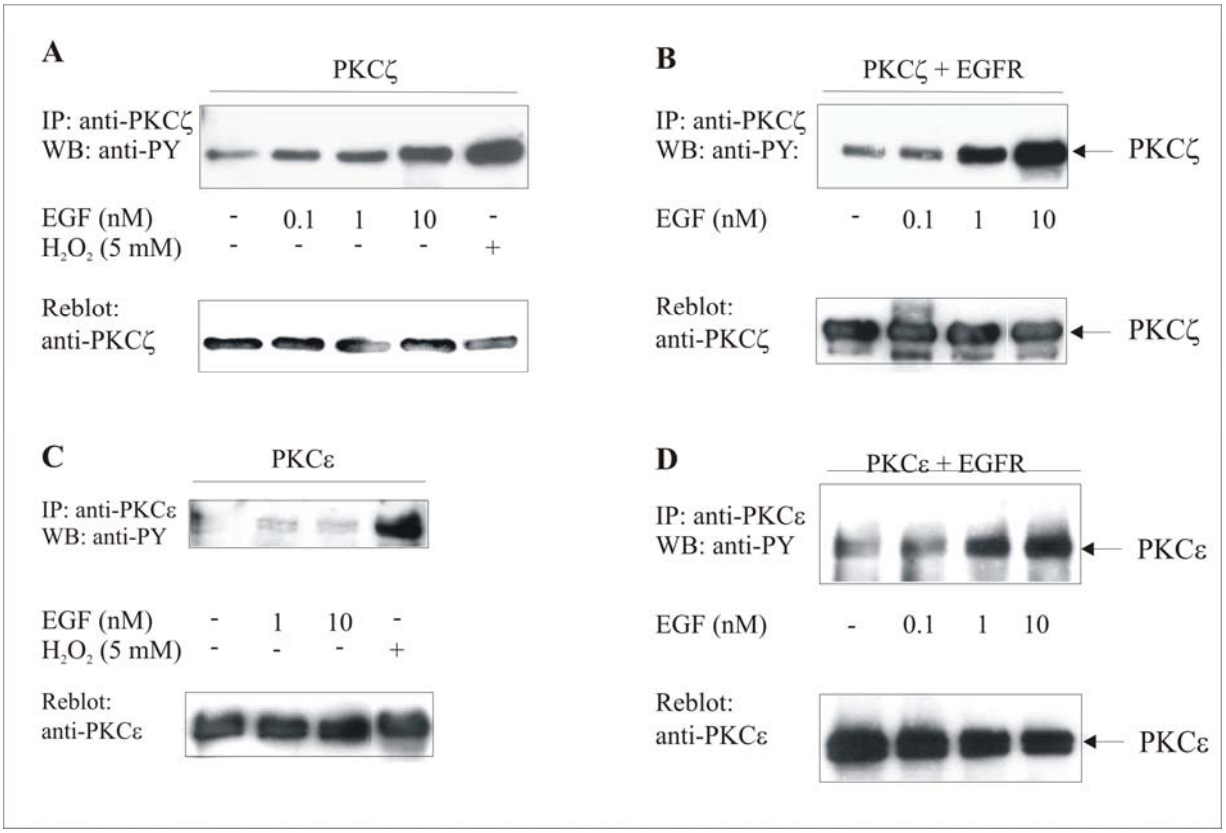


Figure 3.4. EGF induces tyrosine phosphorylation of PKCζ and PKCε in a concentration-dependent maner. COS-7 cells were transiently transfected with PKCζ (A), PKCε (C), co-transfected with PKCζ and EGFR expression vectors (B) or PKCε and EGFR expression vectors (D). Cells were serum starved and then left untreated, treated with indicated EGF concentrations for 5 min or treated with 5mM H_2O_2 for 10 min as a positive control. PKC immunoprecipitates were analysed by Western blotting with PY99 phosphotyrosine-specific antibody and reprobed with PKCζ-specific antibody (A, B) or PKCε-specific antibody (C, D). Results shown are representative of four independent experiments.

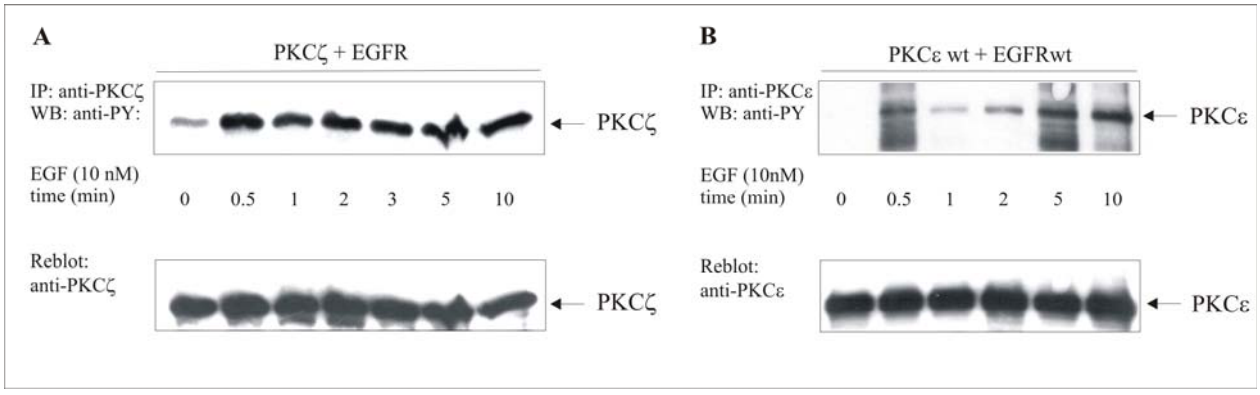


Figure 3.5. Tyrosine phosphorylation of PKCζ and PKCε is early event in response to EGF stimulation. COS-7 cells co-expressing either EGFR and PKCζ (A) or EGFR and PKCε (B) were stimulated with 10nM EGF for indicated time periods. PKC immunoprecipitates were analysed by Western blotting with PY99 phosphotyrosine-specific antibody and reprobed with PKCζ- (A) or PKCε- specific antibody (B). Shown is a representative of two independent experiments.

The time course of the modification of PKCζ and PKCε by tyrosine phosphorylation was followed in COS-7 cells co-expressing EGFR and PKCζ or EGFR and PKCε. Tyrosine phosphorylation of both isoforms can be detected very early- after 30 sec stimulation with 10nM EGF. The level of the tyrosine phosphorylation of PKCζ peaked at this early time point, decreased slightly upon EGF stimulation for 1min, again increased after 2 min and sustained for the prolonged time periods of stimulation - up to 10 min as it is shown on Figure 3.5.A. The level of PKCε tyrosine phosphorylation increased after 30 sec EGF stimulation, showed some decrease upon EGF stimulation for 1min and gradual increased in response to EGF stimulation for 2 –10 min (Figure 3.5.B). Thus it became clear that both PKCζ and PKCε are tyrosine phosphorylated in response to EGFR stimulation in a ligand concentration-dependent manner. Moreover, the modification of both PKC isoforms appeared to be early event upon EGFR stimulation with slightly biphasic patterns and different kinetic of PKCζ and PKCε tyrosine phosphorylation. As the experiments using 5 min stimulation with 1 to 10 nM EGF were best reproducible we have followed this conditions in our further experiments.

3.2.2. Dependence on EGFR tyrosine kinase activity

In order to assess the importance of EGFR tyrosine kinase activity for EGF-induced tyrosine phosphorylation of PKCζ and PKCε we pretreated the cells with 10nM AG1478, an inhibitor of EGFR kinase activity, for 30 min prior stimulation with 10nM EGF for 5 min.

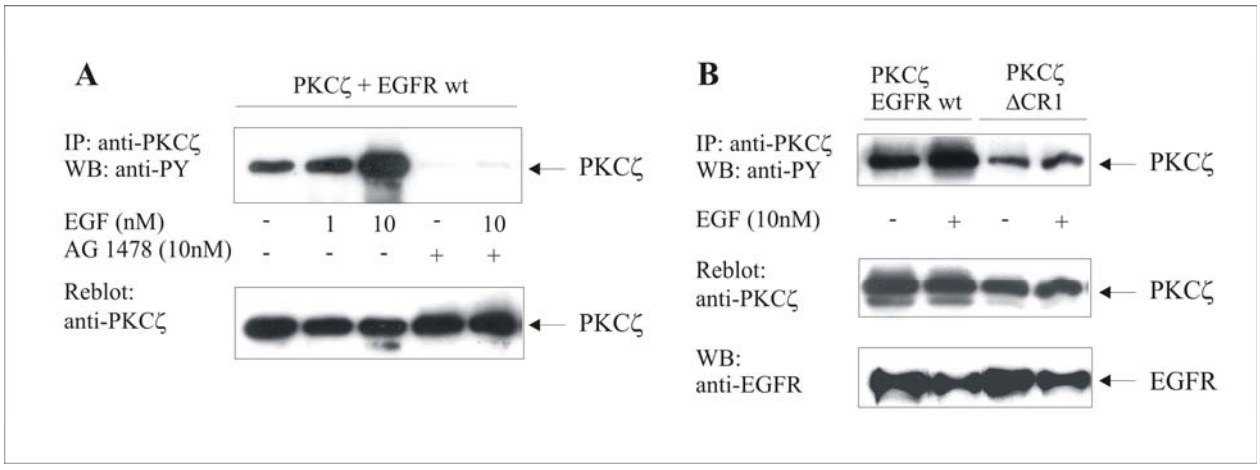


Figure 3.6. Tyrosine phosphorylation of PKCζ is dependent on the kinase activity of the EGFR. (A) COS-7 cells co-transfected with 2μg PKCζ and 1μg wt EGFR were serum starved, pretreated for 30 min with vehicle or 10nM AG1478 and then left untreated or stimulated with EGF as indicated. PKCζ was immunoprecipitated and analysed by Western blotting with anti-phosphotyrosine PY99 antibody and reprobed with PKCζ antibody. (B) COS-7 cells were transfected with 0.3μg PKCζ plus 0.3μg wt EGFR or 0.3μg PKCζ along with 3μg EGFR dimerization mutant ΔCR1. Cells were serum starved and then left untreated or stimulated with EGF as indicated. PKCζ immunoprecipitates were analysed as in (A). Expression levels of EGFR wild type and EGFR ΔCR1 were compared in cell lysates, probed with anti-EGFR antibody 13G8. Results are representative of four independent experiments for AG1478 effect and two independent experiments for ΔCR1 influence.

Inhibition of the EGFR activity by such pretreatment fully abolished the basal as well as EGF-induced tyrosine phosphorylation of both PKCζ (Fig. 3.6.A) and PKCε (not shown). This observation demonstrated that the tyrosine kinase activity of EGFR is critically involved in the tyrosine phosphorylation of both PKC isoforms. From this point the tyrosine phosphorylation of PKCε downstream of EGFR activation became a task for the diploma work of Sabine Maerz.
Additional evidence supporting the role of the receptor tyrosine kinase in PKCζ tyrosine phosphorylation came from the experiment in which we overexpressed a dimerization mutant of EGFR (ΔCR1) instead of the wild type (Figure 3.6.B). This mutant EGFR is not able to dimerize upon ligand stimulation, due to the lack of the entire dimerization arm in domain II (Garrett *et al*., 2002). When the dimerization mutant of EGFR is coexpessed together with PKCζ the basal as well as the EGF-induced tyrosine phosphorylation of PKCζ is significantly reduced, compared to the tyrosine phosphorylation mediated by wild type EGFR. The effect of both AG1478 and EGFR dimerization mutant on the basal tyrosine phosphorylation might be explained by involvement of the ligand-independent basal activity of the overexpressed EGFR. The results obtained upon EGFR dimerization mutant co-expression further supported the suggestion that functional and catalytically active EGFR is required for the observed PKCζ modification on tyrosine residues upon EGF stimulation.

3.2.3. Dependence on Src tyrosine kinase activity

Many recent reports demonstrate involvement of Src family of non-receptor tyrosine kinases in tyrosine phosphorylation of PKC upon number of stimuli. On the other hand Src is activated downstream of EGFR activation. Therefore, we investigated the involvement of Src family tyrosine kinases as possible mediators of EGFR-induced tyrosine phosphorylation of PKCζ. We found that overexpression of wild type Src resulted in a significantly increased level of basal as well as EGF-induced tyrosine phosphorylation of PKCζ (Figure 3.7). Moreover, upon co-transfection of the dominant negative (RF) mutant of Src, both the basal and EGF-induced tyrosine phosphorylation of PKCζ were severely abrogated. Wild type and dominant negative Src were equally expressed. PKCζ and EGFR expression levels were in similar manner reduced upon either wild type or dominant-negative Src co-expression and can not account for the differences observed in the tyrosine phosphorylation. Thereby the effects of wild type and dominant negative Src unequivocally suggest that Src family cytoplasmic tyrosine kinase activity is necessary for the tyrosine phosphorylation of PKCζ downstream of EGFR activation.

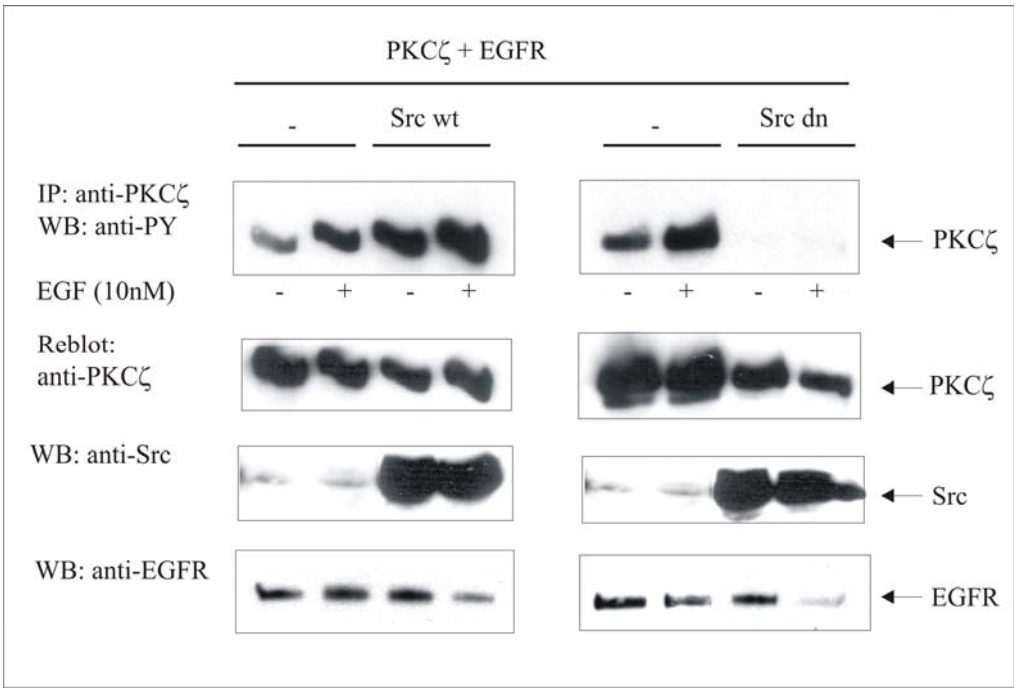


Figure 3.7. PKCζ tyrosine phosphorylation depends on the kinase activity of Src. COS-7 cells were co-transfected with 0,6μg (40 ngDNA/10^5 cells) PKCζ and EGFR and either 0.7μg (50 ngDNA/10^5 cells) wild type Src or 1.2μg (80 ng cDNA/ 10^5 cells) dominant negative Src. Cells were serum-starved and left untreated or EGF stimulated as indicated. PKCζ was immunoprecipitated and analysed by Western immunoblotting with phosphotyrosine-specific antibody PY99 and reprobed with PKCζ-specific antibody. Expression of Src and EGFR were analysed in aliquots of the cell lysates (corresponding to 10^5 cells) using anti-Src antibody (clone 327) and anti-EGFR (13G8) antibody. Similar results were obtained in two independent experiments.

3.2.4. Dependence on PI3K activity and PIP3 production.

Next we used PI3K inhibitors to check the possible involvement of PI3K activity in EGFR-induced tyrosine phosphorylation of PKCζ. As it is shown on Figure 3.8.A the pretreatment either with 25μM LY294002 or 100nM Wortmannin for 30 min led to partial decrease in EGF-induced PKCζ tyrosine phosphorylation. The effect of PI3K inhibitors was less pronounced than the effects of EGFR tyrosine kinase inhibitor, EGFR dimerization mutant or Src dominant negative mutant. This was the indication for partial dependence of PKCζ tyrosine phosphorylation on the activity of PI3K. We suggested that it could be the product of PI3K function PIP3, which is of importance for PKCζ tyrosine phosphorylation downstream of EGFR activation. Therefore, we analysed the influence of co-expression of PTEN- a lipid phosphatase implicated in the removal of phosphate from the third position of PIP3. The effect of PTEN co-expression on EGF-induced PKCζ tyrosine phosphorylation was really strong inhibition (Figure 3.8.B) but since the EGFR is lower expressed the PTEN effect probably is only partial. Upon co-expression of wild type 110beta catalytic subunit of PI3K the EGF-stimulated tyrosine phosphorylation of PKCζ was not positively influenced. Moreover, the basal tyrosine phosphorylation of PKCζ was decreased (data not shown). The results demonstrated partial dependence of PKCζ tyrosine phosphorylation on

the PI3K activity probably based on the need of PIP3 production for recruitment of PKCζ close to the membrane.

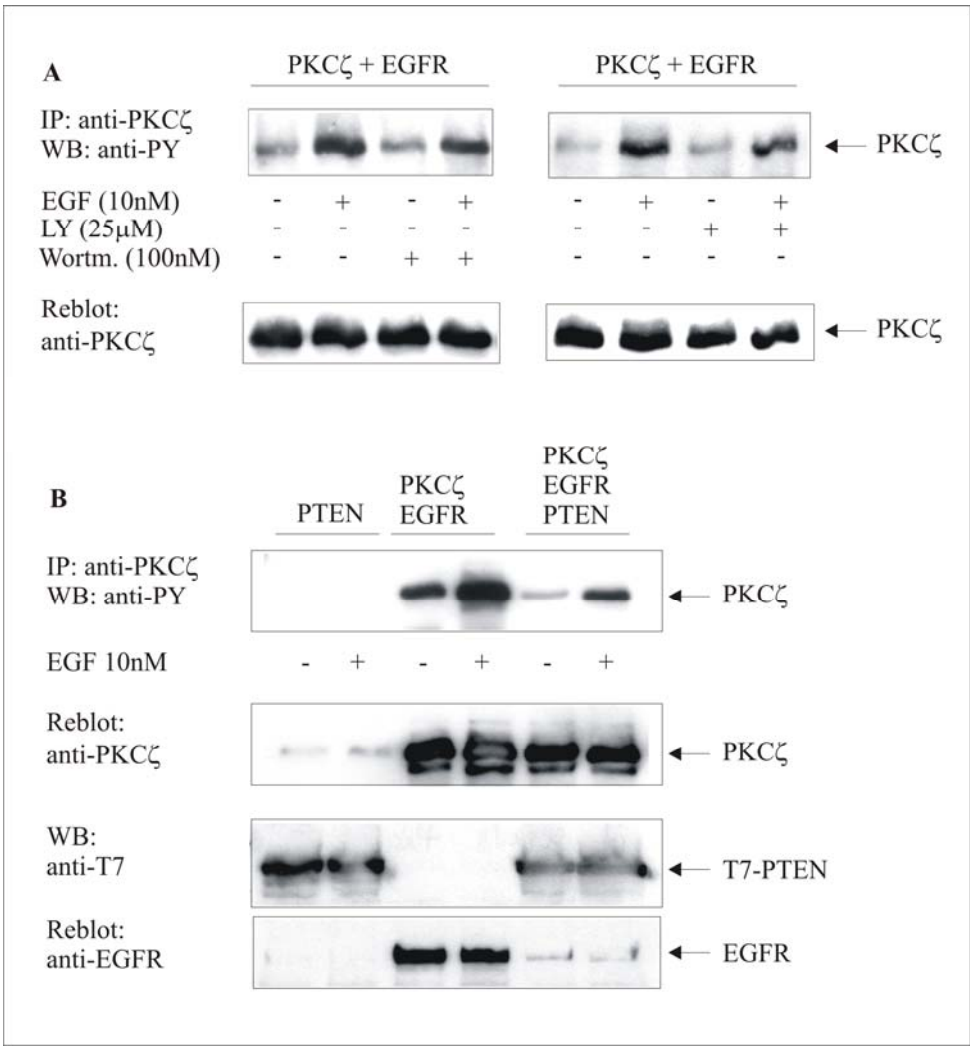


Figure 3.8. PI3K is involved in PKCζ tyrosine phosphorylation downstream of EGFR activation. COS-7 cell were cotransfected with 40ng/10^5 cells PKCζ and EGFR (A), or with 75ng/10^5 cells of each PKCζ, EGFR and PTEN-T7 expression vectors (B). Serum-starved cells were pretreated with vehicle, 25μM LY 294002 or 100nM Wortmannin (A) for 30 min prior to 10nM EGF stimulation for 5 min as indicated. PKCζ was immunoprecipitated and analysed by Western blotting with phosphotyrosine-specific antibody 4G10 (A) or PY99 (B) and reprobed with PKCζ-specific antibody. Expression of T7-PTEN and EGFR were analysed in aliquots of the cell lysates (corresponding to 10^5 cells) using anti-T7 tag antibody and anti-EGFR (13G8) antibody. Similar results were obtained in two independent experiments.

3.2.5. Involvement of the tyrosine residues in the cytoplasmic part of the EGFR

Further we tried to identify which of the tyrosine residues in the cytoplasmic part of EGFR are required for tyrosine phosphorylation of PKCζ to occur. We co-expressed PKCζ together with EGFR mutants each possessing a substitution of distinct tyrosine residue serving as a docking site in its phosphorylated state to unphosphorylatable phenylalanine residue. In this way we attempted to estimate first whether the activation of distinct signalling cascades downstream of EGFR

contributes to PKCζ tyrosine phosphorylation and second, whether some of these docking sites are involved in assembling the proteins involved in PKCζ tyrosine phosphorylation. Phosphotyrosine 992 is a direct binding site for the phospholipase C-γ (PLC-γ) SH2 domain. Phosphotyrosine 1173 represents an additional docking site that couple EGFR to PLC-γ pathway. Via Gab1 and p85 recruitment to phosphotyrosine 1086 EGFR activation is linked to activation of Akt/PKB cascade (Figure 1.1.). In contrast to these autophosphorylation sites tyrosine 845 in the activation loop of EGFR represents Src-phosphorylation site. In our cell model we must always consider the function of endogenous EGFR in COS-7 cells, which is not impaired by the overexpression of different EGFR mutants and could account for at least part of the observed PKCζ tyrosine phosphorylation. Coexpression of Y1173F Y992F, and Y1086F EGFR mutants influenced PKCζ tyrosine phosphorylation state in a positive, rather that in a negative manner (Figure 3.9.A).

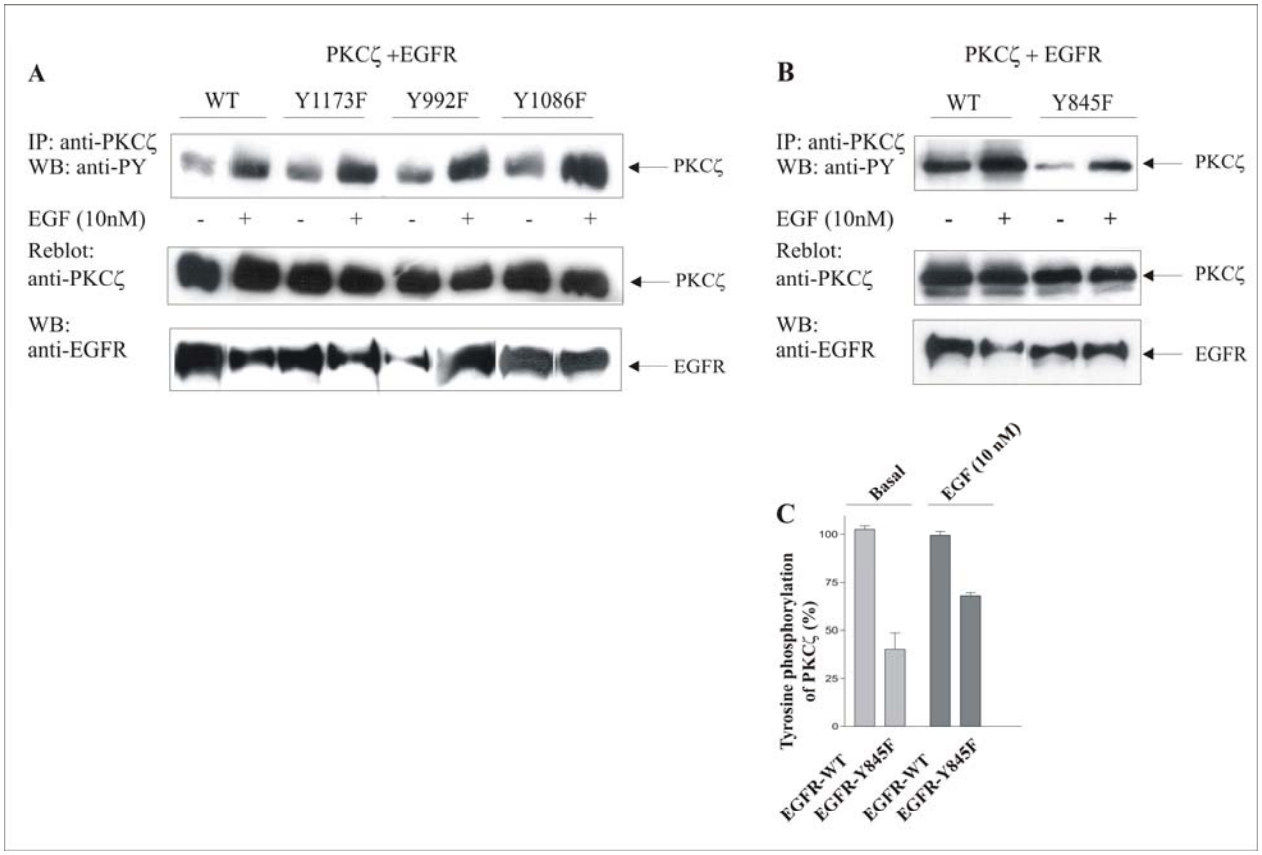


Figure 3.9. Role of tyrosine phosphorylation sites of EGFR on EGFR-induced tyrosine phosphorylation of PKCζ. COS-7 cells were cotransfected with 100ng/10^5 cells PKCζ and EGFR expression constructs WT, Y1173F, Y992F or Y1086F (A) or with 40ng/10^5cells PKCζ and wild type EGFR or 260ng /10^5cells EGFR Y845F mutant (B). Serum-starved cells were treated with 10nM EGF as indicated. PKCζ was immunoprecipitated and analysed by Western blotting with phosphotyrosine-specific antibody PY99 and reprobed with PKCζ-specific antibody. Expression of WT EGFR and EGFR mutants were analysed in aliquots of the cell lysates (corresponding to 10^5 cells) using anti-EGFR (13G8) antibody. Similar results were obtained in two (A) independent experiments. (C) PKCζ tyrosine phosphorylation in cells expressing EGFR Y845F related to the cells expressing wild type EGFR. The results are expressed as means ± standard errors from 5 independent experiments. A representative one is shown in (B).

EGF-induced PKCζ tyrosine phosphorylation in cells coexpressing one of the listed above EGFR mutants was even higher compared to the wild type EGFR-mediated one. Interestingly, upon coexpression of Y845F mutant of EGFR, tyrosine phosphorylation of PKCζ is significantly diminished compared to the modification observed upon coexpression of wild type EGFR (Figure 3.9.B). Quantification of the reduction in PKCζ tyrosine phosphorylation upon expression of EGFR Y845F mutant revealed that its basal phosphorylation is 40% and the EGF-stimulated increase is 65 % of the respective levels in cells that express wild type EGFR (Figure 3.9.C). This suggests that phosphorylation of tyrosine 845 is necessary for the PKCζ tyrosine phosphorylation downstream of EGFR. As long as it has been demonstrated that tyrosine 845 of EGFR is Src phosphorylation site this is further evidence for the functional involvement of Src in EGFR-mediated PKCζ tyrosine phosphorylation.

3.2.6. Tyrosine phosphorylation of PKCζ and PKCε in correlation with its activity state

Another interesting point was to adrress the relation of the modification of PKCζ by tyrosine phosphorylation to the activity state of the enzyme. We assessed that by comparison of the EGFR-mediated tyrosine phosphorylation of wild type, constitutively active and kinase inactive PKCζ or PKCε. Constitutively active mutants of both PKC isoforms possess mutation of Ala to Glu in the pseudosubstrate region, which prevents pseudosubstrate inhibiton (Decock *et al*., 1994). The kinase inactive mutants harbor substitution of critical Lys residue to Arg at the ATP binding site of the kinase domain (Jaken *et al*., 1996). Despite that COS-7 cells express endogenously PKCζ, whose tyrosine phosphorylation masks that of the respective mutants, we found significant and reproducible differences between their levels of tyrosine phosphorylation. Constitutively active mutant of PKCζ is less tyrosine phosphorylated compared to the wild type enzyme, whereas the inactive mutant of PKCζ is significantly higher tyrosine phosphorylated in response to EGF stimulation (Figure 3.10.A). The differences were even more pronounced in the case of the respective mutants of PKCε (Figure 3.10.B). The absolute increase in the EGF-induced tyrosine phosphorylation of both PKCζ and PKCε inactive species is more significant when we consider their lower expression levels. In addition, using different ratios of the expression vectors for PKCζ and EGFR during transfection we found that when the PKCζ : EGFR ratio is 2:1 the tyrosine phosphorylation of PKCζ is lower compared to the modification observed under 1:1 ratio of both constructs (not shown). These results indicated that the activity state or the relative amount of PKCζ influences its EGFR-mediated tyrosine phosphorylation might reflect the negative feedback regulation of EGFR tyrosine kinase activity probably by PKC-mediated threonine phosphorylation.

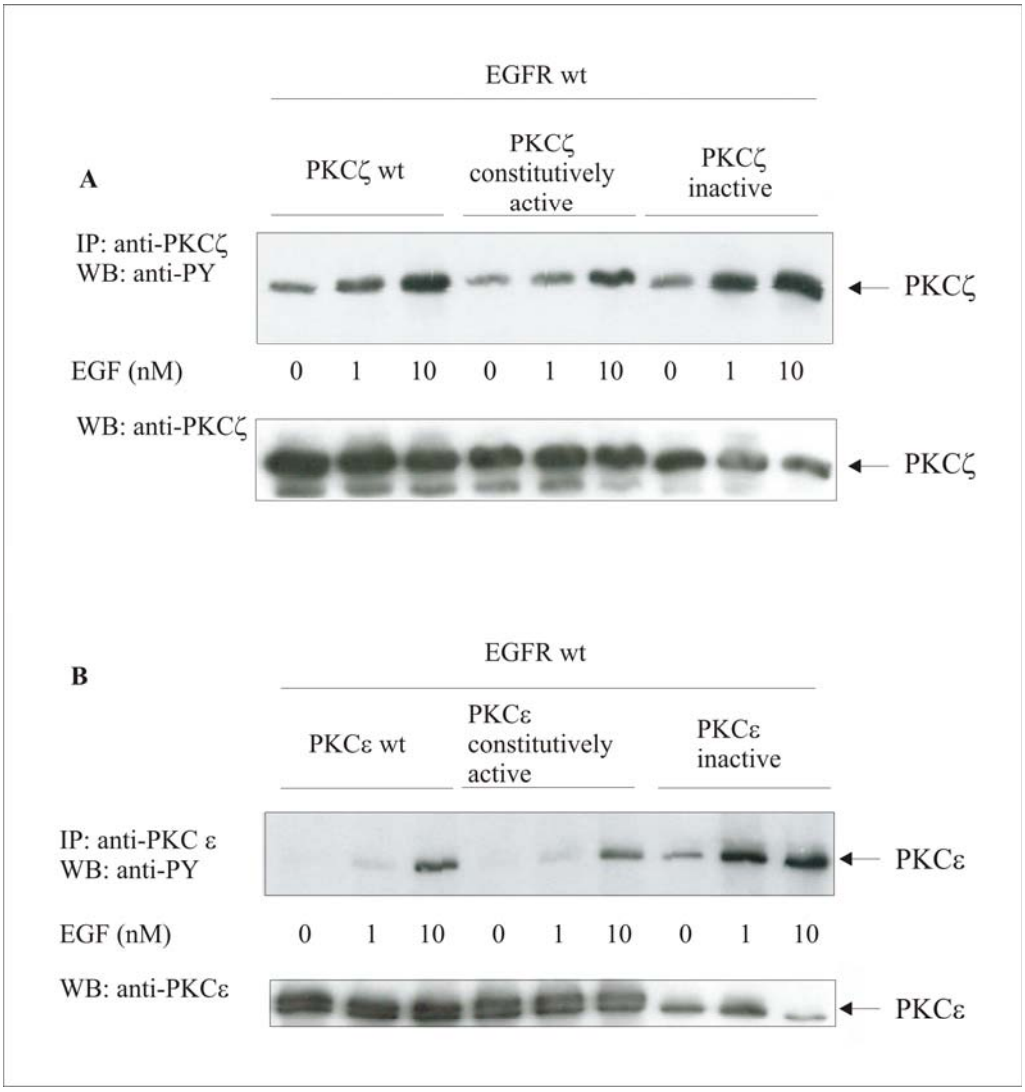


Figure 3.10. Tyrosine phosphorylation of PKCζ and PKCε is dependent on PKC activity state. COS-7 cells were cotransfected with 60ng/10^5 cells EGFR and 125ng/10^5 cells of WT, constitutive active, or inactive PKCζ expression constructs (A), or WT, constitutive active, or inactive PKCε (B). Serum-starved cells were stimulated with 1nM and 10nM EGF as indicated. PKCs were immunoprecipitated and analysed by Western blotting with anti-phosphotyrosine PY99 antibody and reprobed with PKCζ (A) or PKCε (B). Similar results were obtained in two independent experiments.

3.3. Interaction of PKCζ and EGFR

3.3.1. Co-immunoprecipitation

In the course of our investigation on the EGFR-mediated PKCζ tyrosine phosphorylation we identified a co-immunoprecipitation of tyrosine phosphorylated PKCζ with another tyrosine phosphorylated protein with apparent molecular weight about 170-180 kDa (Figure 3.11.A). Furthermore, the reprobing with EGFR-specific antibody revealed that the tyrosine-

phosphorylated band could be identified as EGFR, thus indicating for direct or indirect interaction between the EGFR and PKCζ.

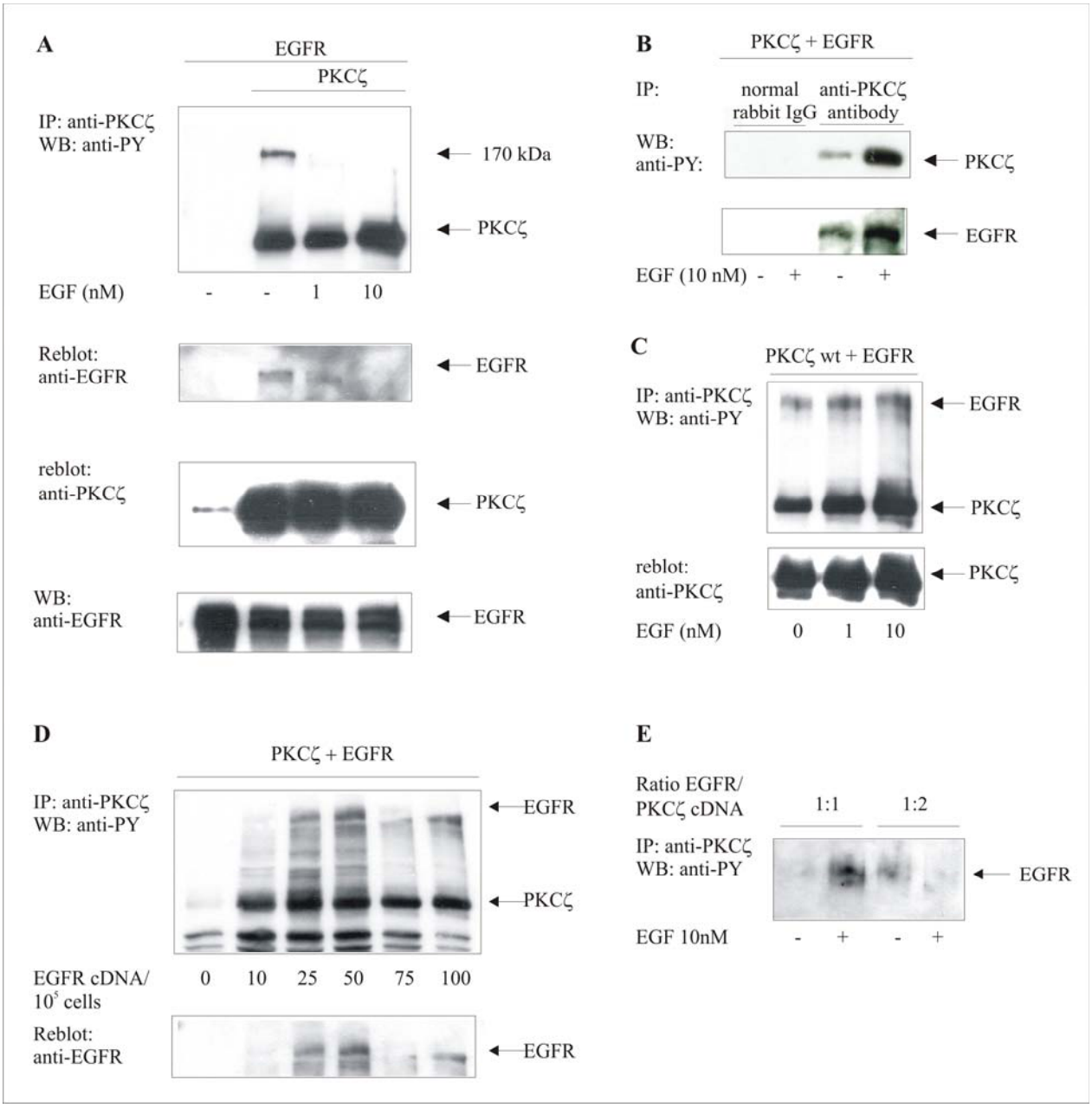


Figure 3.11. Co-immunoprecipitation of EGFR and PKCζ. COS-7 cells were transfected with 75ng/10^5 cells EGFR expression construct or cotransfected with 150ng/10^5 cells PKCζ and 75ng/10^5 cells EGFR (A), or with 100 ng/10^5 cells of PKCζ and 100 ng/10^5 cells EGFR (B and C), with 120ng/10^5 cells PKCζ along with different amount of EGFR as indicated (D), with 100ng/10^5 cells PKCζ and 100ng/10^5 cells EGFR for 1:1 ratio or 135ng/10^5 cells PKCζ and 65ng/10^5 cells EGFR for 2:1 ratio upon transfection (E). Serum-starved cells were stimulated with EGF as indicated. PKCζ immunoprecipitates or control immunoprecipitates with normal rabbit immunoglobulin (B) were analysed by Western blotting with phosphotyrosine-specific antibody PY99 and reprobed with EGFR-specific antibody (A, D) and PKCζ-specific antibody (A, B and C). Expression of WT EGFR was analysed in aliquots of the cell lysates (corresponding to 10^5 cells) using anti-EGFR (13G8) antibody (A). The results are respresentative of three independent experiments.

In parallel lane immunoprecipitate of endogenous PKCζ from EGFR overexpressing COS-7 cell shows no unspecific reactivity of the PKCζ antibody to the highly expressed EGFR. The specificity of the co-immunoprecipitation of PKCζ with EGFR was further tested by the use of normal rabbit immunoglobulin as a negative control in the immunoprecipitation of cell lysates from cells co-transfected with EGFR and PKCζ. The co-immunoprecipitation was observed only in the PKCζ-specific antibody immunoprecipitates but not upon immunoprecipitation performed with the same amount of normal rabbit immunoglobulin (Figure 3.11.B). The first result indicating co-immunoprecipitation (Figure 3.11.A) showed that more EGFR is found in PKCζ immunoprecipitates in the non-stimulated cells. Upon stimulation with 1nM and 10nM EGF the amount of EGFR co-immunoprecipitating with PKCζ as well as its tyrosine phosphorylation were clearly reduced. These patterns of the tyrosine phosphorylation of the EGFR in PKCζ immunoprecipitates were not reproduced in all further experiments (Figure 3.11. A and B). More typical result was as it is shown on Figure 3.11.C- EGFR interacting with PKCζ under non-stimulated conditions was lower tyrosine phosphorylated, whereas EGFR in PKCζ immunoprecipitates from cells treated for 5 min with 1-10nM EGF was highly tyrosine phosphorylated. In order to explain these different patterns of co-immunoprecipitation we compared the tyrosine phosphorylation of the EGFR in PKCζ immunoprecipitates under non-stimulated conditions in relation to the amount of EGFR expression vector used for transfection. We observed that the co-immunoprecipitation was more pronounced in the cells transfected with lower amount EGFR 25-50ng EGFR per 10^5 cells and respectively decreased upon transfection of more 75-100ng/10^5 cells (Fig.3.11.D). In addition, the detection of co-immunoprecipitation between EGFR and PKCζ showed dependency on the ratio of both expression constructs used for transfection, thereby dependent on the stoichiometric ratio of both expressed proteins, similarly to PKCζ tyrosine phosphorylation. When both vectors are used during transfection in the ratio 1:1 (1.5 μg : 1.5 μg) 10nM EGF stimulation leads to increased tyrosine phosphorylation of the EGFR in PKCζ immunoprecipitates. In contrast when the transfection ratio was 1:2 (1μg EGFR: 2 μg PKCζ) the tyrosine phosphorylation of the EGFR co-immunoprecipitating with PKCζ was higher under basal conditions (Figure 3.11.E). Therefore small changes in the plasmid DNA amount and ratio used in different transfections could explain the variations we found in the co-immunoprecipitation patterns.

Under completely endogenous conditions no co-immunoprecipitation was found in lysates from the typical number of COS-7 cells used in our experiments (1.4-1.6 x 10^6). Using lysates from 3 x 10^6 cells stimulated with 1nM EGF PKCζ was identified in EGFR immunoprecipitates (Figure 3.12.A). Vice versa EGFR has been found in PKCζ immunoprecipitates upon EGF stimulation but not in normal rabbit IgG immunoprecipitates.

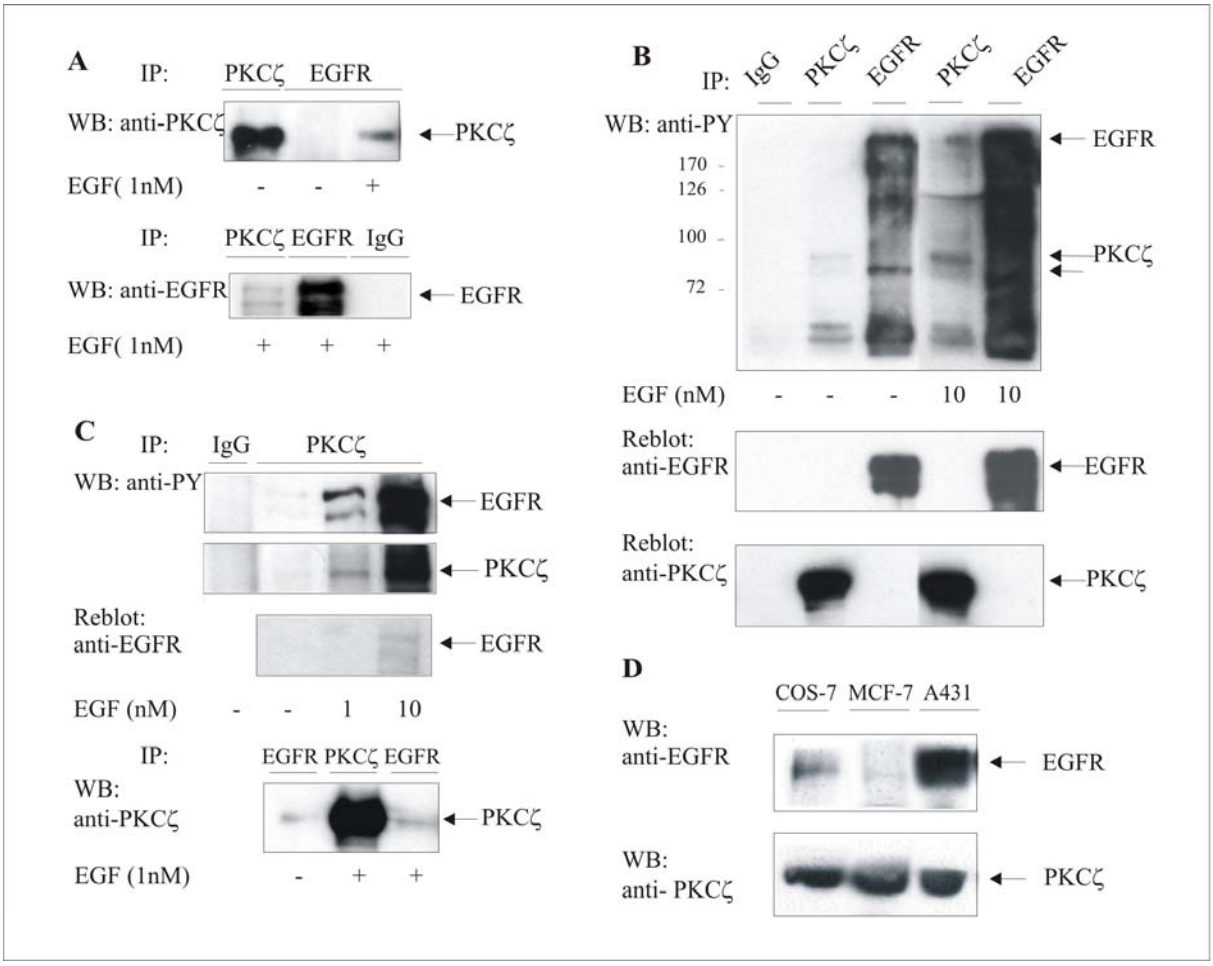


Figure 3.12. Co-immunoprecipitation of EGFR and PKCζ under endogenous conditions. 3 x 10^6 COS-7 cells (A) or 1.5 x 10^6 A431 (B) and MCF-7 cells (C) untreated or stimulated with EGF as indicated were lysed. Parallel immunoprecipitations with EGFR-specific antibody, PKCζ-specific antibody, or normal rabbit immunoglobulin were carried on. Immunoprecipitates were analysed by Western blotting with phosphotyrosine-specific antibody PY99 and reprobed with either EGFR-specific antibody 13G8 or PKCζ-specific antibody. Expression level of EGFR and PKCζ were analyzed in aliquots of the cell lysates (corresponding to 10^5 cells) using EGFR-specific antibody (13G8) and PKCζ-specific antibody (D). Similar results were obtained in two independent experiments.

In epidermoid carcinoma cell line A431 harboring highly overexpressed EGFR (10^6 receptors/cell) PKCζ seemed to be only faintly tyrosine phosphorylated upon stimulation with 10nM EGF. Figure 3.12.B shows that neither non-immune IgG nor PKCζ-specific antibody recognizes EGFR despite of its huge amount. Only the phosphotyrosine-specific antibody detected a band on its position in PKCζ immunoprecipitates from cells stimulated with 10nM EGF. In EGFR immunoprecipitates other tyrosine-phosphorylated protein with slightly lower molecular weight than PKCζ was identified. Obviously, if any interaction between EGFR and PKCζ occurs in A431 cells, it is detectable only after 10nM EGF stimulation and only in one direction- EGFR in PKCζ immunoprecipitates but not vice versa. Breast carcinoma cell line MCF-7 has normal levels of EGFR (1.5 x 10^4 receptors/cell). As it is shown on Figure 3.12.C EGFR was detected in PKCζ immunoprecipitates and vice versa PKCζ was found in EGFR

immunoprecipitates. In addition PKCζ was tyrosine phosphorylated upon EGF stimulation. These results indicate that both the interaction of EGFR and PKCζ and EGFR-induced tyrosine phosphorylation of PKCζ take place under endogenous conditions and could be of some importance in the signal transduction of cancer cell lines. Figure 3.12.D shows that PKCζ expression in COS-7, A431 and MCF-7 is similar, whereas they express different amount EGFR-low in MCF-7 and COS-7 cells and highly overexpressed in A431.

In COS-7 cells transfected with both PKCζ and EGFR we not only found EGFR in PKCζ immunoprecipitates but we also identified vice versa PKCζ in EGFR-specific antibody immunoprecipitates. The time course of the interaction between PKCζ and EGFR is shown on Figure 3.13. The co-immunoprecipitation was detected under basal conditions in both directions and both EGFR in PKCζ immunoprecipitates as well as PKCζ in EGFR immunoprecipitates showed an increase after short-term stimulation with 10nM EGF (30 sec). The EGFR detected in the PKCζ immunoprecipitates dropped to the basal amount for stimulation times ranging from 1 min up to 5 min. The amount of PKCζ detected in the EGFR immunoprecipitates is still high after 1 min stimulation and decreased during 3 to 15 min long EGF stimulation time. A second phase of increased co-immunoprecipitation was observed between 10 and 30 min for EGFR in the PKCζ immunoprecipitates and after 20 min EGF stimulation for PKCζ in EGFR immunoprecipitates, respectively. There is good correlation of the time course of the vice versa co-immunoprecipitations with some shift for PKCζ in EGFR immunoprecipitates to the late time points. The threonine phosphorylation of EGFR followed PKCζ presence in the EGFR immunoprecipitates showing corresponding time-dependent changes with some increase between 1 and 5 min, decrease after 10 to 15 min and further increase after 20 min EGF stimulation (Figure 3.13.A). The only difference is that while upon 3-5 min EGF stimulation PKCζ co-immunoprecipitation with the EGFR decreases, EGFR threonine phosphorylation remained elevated. PKCζ tyrosine phosphorylation is significantly increased after 30 sec EGF stimulation and remained at this elevated level for the whole time period studied (Figure 3.13.B). Tyrosine phosphorylation of EGFR in PKCζ immunoprecipitates was correspondent to the amount of EGFR. It is worth to point that from 1 up to 5 min EGF stimulation less EGFR with elevated threonine phosphorylation interacts with PKCζ. Endogenous EGFR was detected in immunoprecipitates of overexpressed PKCζ and vice versa overexpressed PKCζ was detected in endogenous EGFR immunoprecipitates (Figure 3.13.C). We found that wild type PKCζ interaction with EGFR is slightly increased upon 10nM EGF stimulation for 5 min. Constitutively active PKCζ was found in high amount in EGFR co-immunoprecipitates under basal conditions and the interaction decreased in response to EGF stimulation, whereas the interaction of inactive PKCζ with the EGFR is significantly lower. It seems that the inactive

mutant of PKCζ, which is higher tyrosine-phosphorylated, interacts with the EGFR to a less extent, compared to the less tyrosine-phosphorylated constitutively active PKCζ.

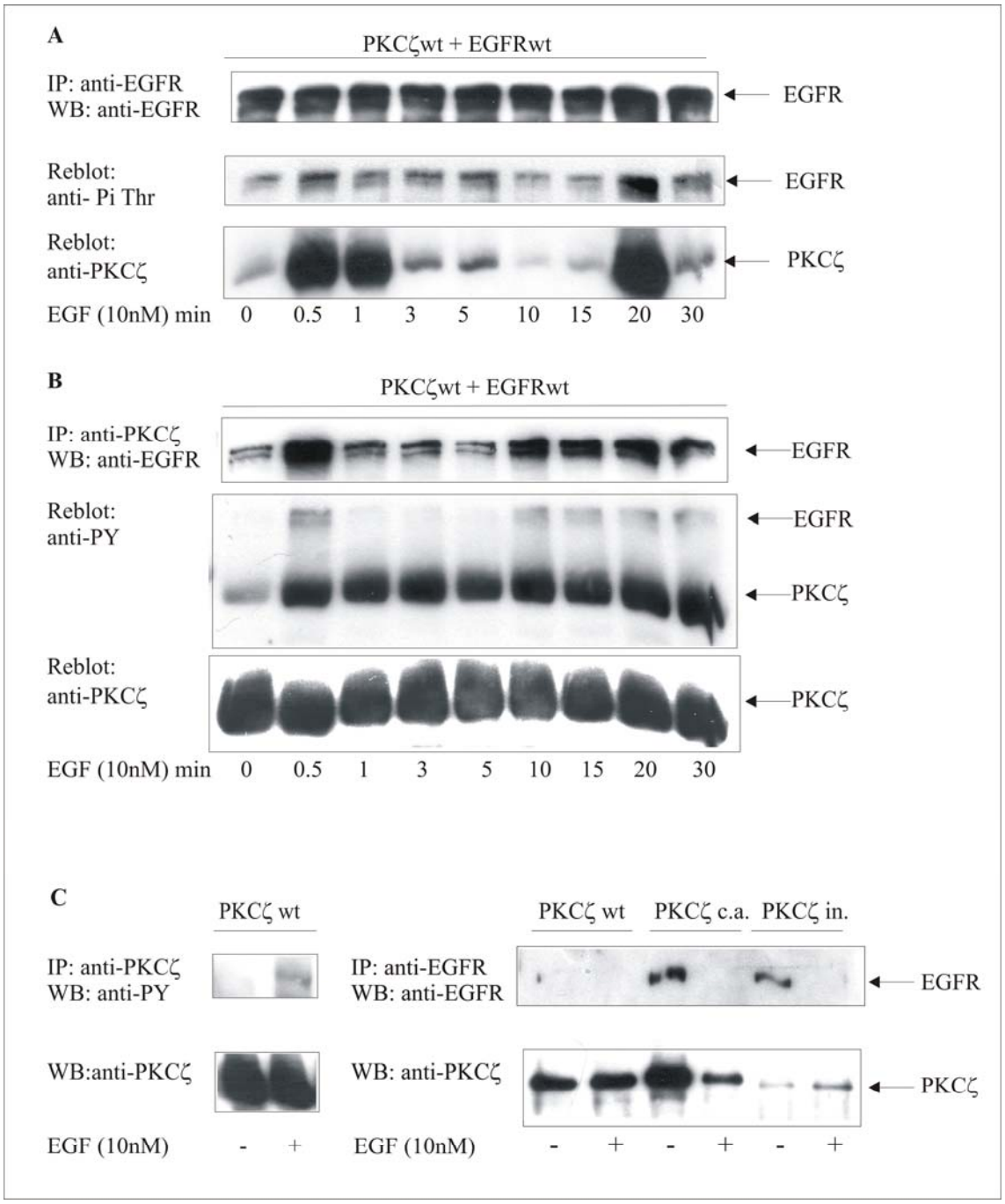


Figure 3.13. coIP vice versa and time course of the coIP. COS-7 cells were cotransfected with 100 ng/10^5 cells PKCζ and 100 ng/10^5 cells EGFR expression vectors (A, B) or transfected with 100ng/10^5 cells either wild type, constitutively active, or inactive mutant of PKCζ (C). Serum-starved cells were stimulated with 10nM EGF for different time periods as indicated (A, B) or for 5 min (C). Immunoprecipitations were performed with EGFR-specific antibody clone 425 (A) or from Santa cruz (C) and with PKCζ-specific antibody (B, C). The immunoprecipitates were analysed by Western blotting with EGFR-specific antibody 13G8 (A, B, C) or phosphotyrosine-specific antibody PY99 (C) and reprobed with phosphothreonine-specific and PKCζ-specific antibody (A) phosphotyrosine-specific antibody and PKCζ-specific antibody (B) or with PKCζ-specific antibody (C). Similar results were obtained in two independent experiments.

This might indicate that the presence of PKCζ in EGFR signalling complex is related to the PKC-dependent threonine phosphorylation of EGFR, but is not directly related to the level of EGFR-induced tyrosine phosphorylation of PKCζ. Thus we can hypothesize that PKCζ tyrosine phosphorylation takes place via indirect interaction with the EGFR, probably through some adaptor protein.

Next we analysed the co-immunoprecipitation of PKCζ with different EGFR mutants. As it is shown on Figure 3.14.A all EGFR mutants were found to co-immunoprecipitate with PKCζ but in different manner. In this experiment the interaction of the wild type EGFR with PKCζ was higher under the unstimulated conditions and decreased upon EGF stimulation (1-10nM). The same were the patterns of the co-immunoprecipitation with PKCζ of EGFR mutants Y992F and Y1086F. The overall amount of EGFR Y1086F in PKCζ immunoprecipitates is less compared to that of wild type EGFR and EGFR Y992F. These differences cannot be explained with different expression of EGFR mutants. Western blot analysis of aliquots from the same lysates with EGFR-specific antibody revealed that different EGFR mutants have equal expression levels (Figure 3.14.A). Since tyrosine 1086 is involved in PI3K activation our finding could indicate that functional activation of PI3K pathway downstream of EGFR is important for the interaction of PKCζ with the EGFR- probably mediated via PIP3 generation and PIP3-dependent recruitment of PKCζ to the membrane compartment, which increase the probability for the interaction with the EGFR. The interaction of EGFR Y992F with the PKCζ is to some extent more pronounced than that of the EGFR wild type indicating probably for some competition between PKCζ and PLCγ for interaction with EGFR. Moreover, the EGFR mutant harboring substitution of the PLCγ and SHP1-docking tyrosine 1173 to phenylalanine showed EGF-stimulation-dependent increase of the co-immunoprecipitation with PLCγ. This suggests that upon EGF-stimulation and in the absence of bound PLCγ and or SHP1 the interaction of EGFR and PKCζ is favored. Interestingly, in correlation with the lower EGF-induced tyrosine phosphorylation of PKCζ upon expression of EGFR mutant Y845F, the amount of the EGFR co-immunoprecipitating with PKCζ is very low. This finding demonstrates the critical role for the phosphorylation of tyrosine 845 on EGFR and probably the activated downstream of phosphotyrosine 845 signalling pathways in the interaction between EGFR and PKCζ. Figure 3.14.B shows that in addition to tyrosine 845, EGFR dimerization is also required for the interaction of both molecules. Demonstration for that is the diminished co-immunoprecipitation of the EGFR dimerization mutant with PKCζ. Both EGFR mutants, which showed diminished PKCζ tyrosine phosphorylation, have also impared co-immunoprecipitation with PKCζ. Figure 3.14.C represents quantification of the decrease in the co-immunoprecpitation of EGFR Y845F with PKCζ in comparison to the wild type EGFR. Substitution of tyrosine 845 of EGFR to phenylalanine caused around 45 % decrease in the interaction with PKCζ.

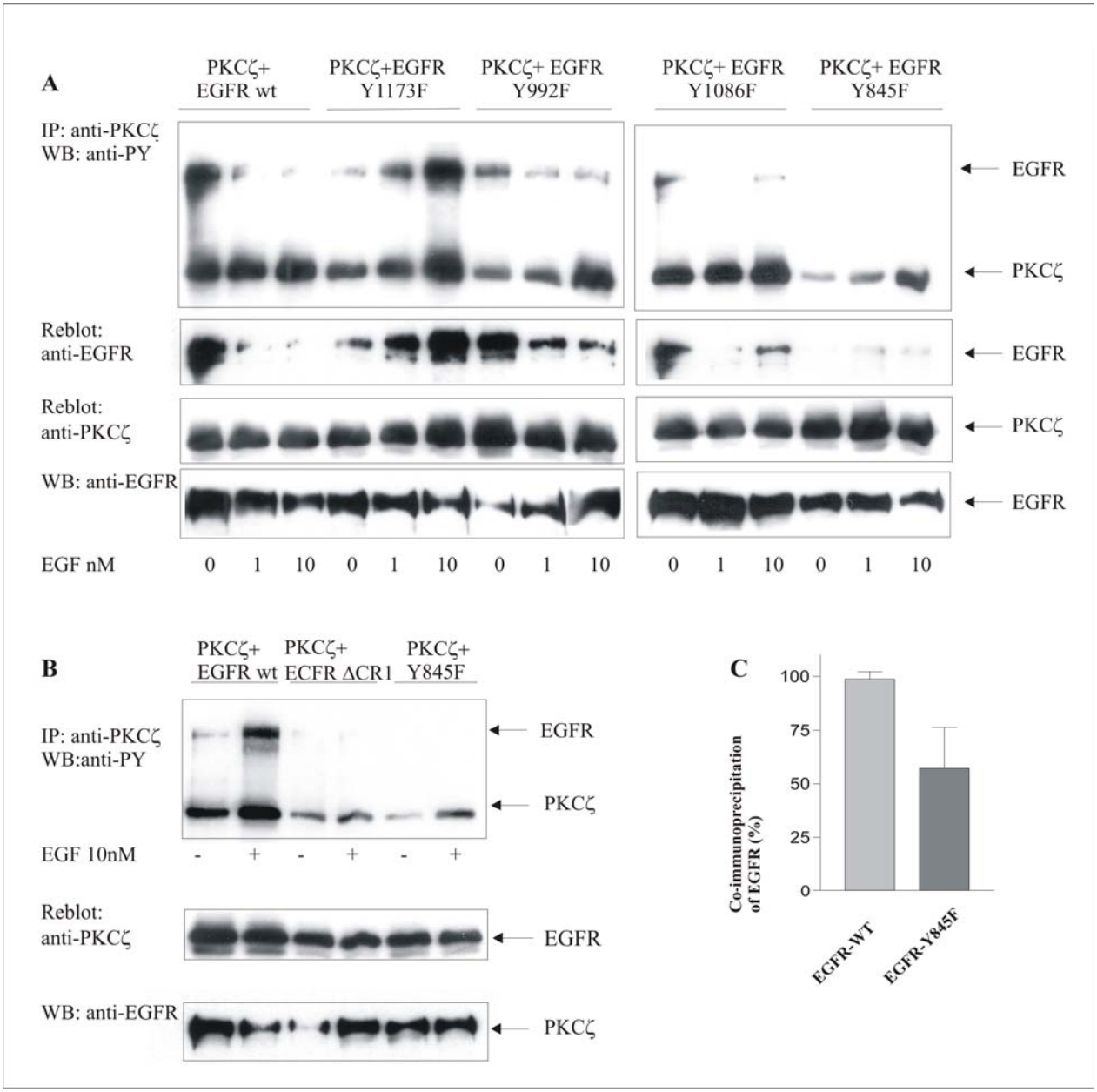


Figure 3.14. Co-immunoprecipitation of EGFR mutants with PKCζ. COS-7 cells were cotransfected with 40ng/10^5 cells PKCζ along with either 40ng/10^5 cells EGFR wild type, Y1173F, Y992F, Y1086F or 270ng/10^5 cells EGFR Y845F (A); with 20ng/10^5 cells PKCζ along with either 20ng/10^5 cells wild type EGFR, 200ng/10^5 cells EGFR ΔCR1, or 200ng/10^5 cells EGFR Y845F (B). Serum-starved cells were stimulated with 1-10nM EGF as indicated. PKCζ was immunoprecipitated and immunoprecipitates were analysed by Western blotting with anti-phosphotyrosine antibody PY99 and reprobed with EGFR-specific antibody 13G8 (A), and PKCζ-specific antibody (A and B). Expression of WT EGFR and EGFR mutants were analysed in aliquots of the cell lysates (corresponding to 10^5 cells) using anti-EGFR (13G8) antibody. Similar results were obtained in two independent experiments for all mutants. In (C) co-immunoprecipitation of EGFRY845F with PKCζ as the mean ± SEM from five independent experiments is expressed as the percentage of the co-immunoprecipitation of EGFR-WT with PKCζ.

Next we addressed the importance of PI3K function for the interaction of EGFR and PKCζ. Pretreatment with PI3K inhibitor wortmannin had no influence on the basal interaction, but led to abolishment of the EGF stimulation-induced increase in the interaction (Figure 3.15.A).

Therefore, EGFR-mediated activation of PI3K probably via PIP3 production contributes to the recruitment of PKCζ to the receptor complex. Moreover, co-expression of p110beta subunit of PI3K caused dramatic decrease in EGFR co-immunoprecipitation with PKCζ (Figure 3.15.B). When instead of wild type 110beta the respective kinase dead mutant was co-expressed, there was no influence on the EGFR co-immunoprecipitation with PKCζ. Figure 3.15.C shows that there is interaction of endogenous 110beta with overexpressed PKCζ under unstimulated conditions, which is not detectable upon stimulation with 10nM EGF.

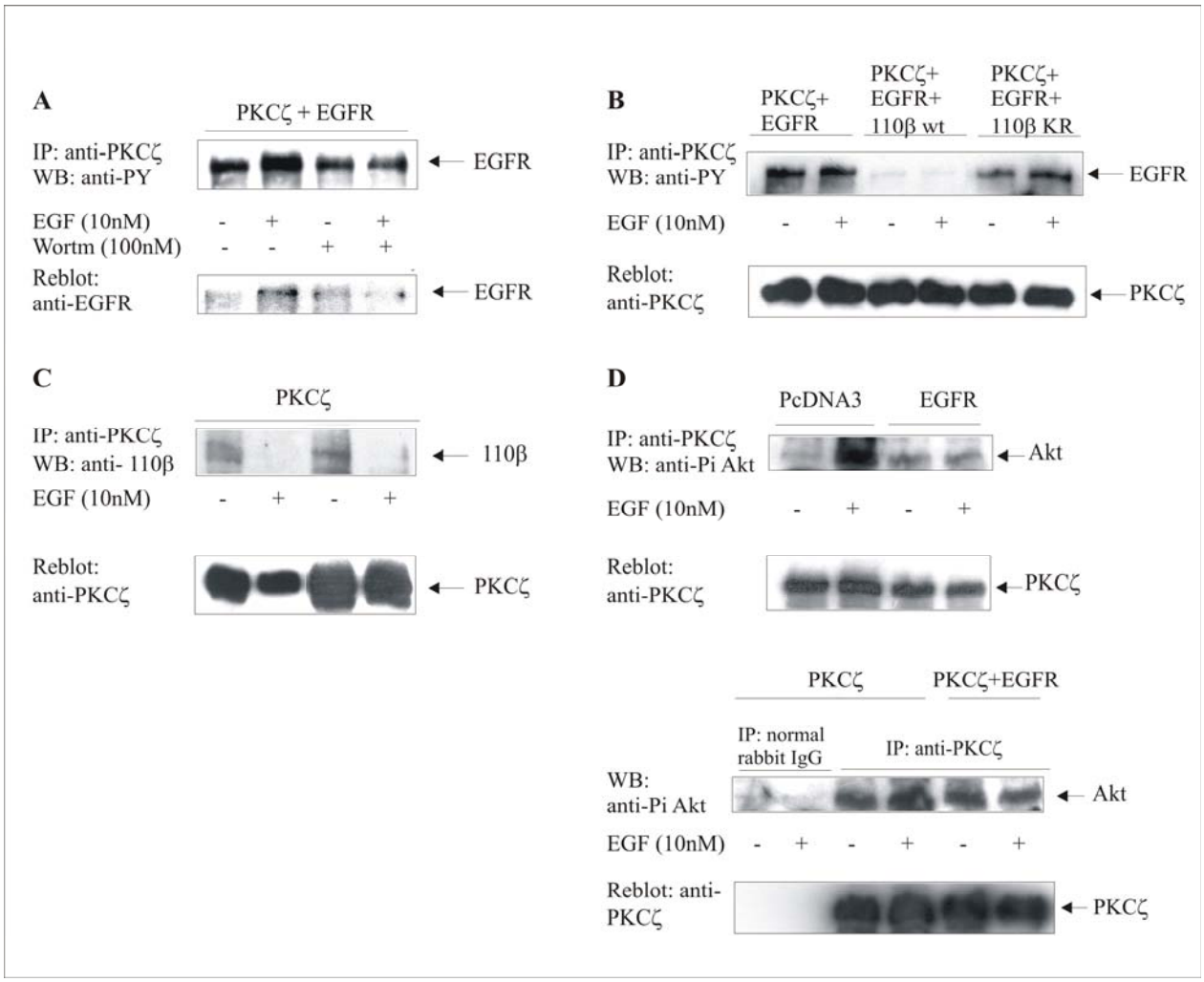


Figure 3.15. Influence of the PI3K signalling pathway on the interaction of PKCζ with the EGFR. COS-7 cells were cotransfected with 40ng/10^5 cells PKCζ along with 40ng/10^5 cells EGFR expression constructs (A); with 100ng/10^5 cells PKCζ along with 100ng/10^5 cells EGFR (B and D) or with 70ng/10^5 cells each PKCζ, EGFR and either 110 beta wild type, or 110 beta KR mutant (B); with 125ng/10^5 cells PKCζ (C). Serum-starved cells were pretreated with 100nM Wortmannin for 30 min (A) and stimulated with 10nM EGF for 5 min as indicated. PKCζ was immunoprecipitated and the immunoprecipitates were analysed by Western blotting with phospho-tyrosine antibody 4G10 (A), PY99 (B), PI3K 110 beta subunit-specific antibody (C), or phospho-Akt-specific antibody (D) and reprobed with EGFR-specific antibody 13G8 (A) or PKCζ-specific antibody (B, C and D). Similar results were obtained in two independent experiments.

Another potential interacting partner of PKCζ in the PI3K-dependent pathway is Akt/PKB. As it is shown on figure 3.15.D in COS-7 cells such interaction occurs and is stimulated under complete endogenous conditions by EGF but is reduced upon EGFR overexpression. PKCζ co-immunoprecipitation patterns of both 110beta and Akt may reflect interaction of PKCζ with 110beta and Akt under basal conditions and recruitment of PKCζ from PI3K complex to EGFR signalling complex in response to EGF stimulation. EGFR and PKCζ co-immunoprecipitated vice versa upon overexpression in COS-7 cells, as well as under endogenous conditions and this finding was indicative for their direct or indirect interaction. EGFR stimulation caused transient increase in the association. Moreover, the interaction seems to be dependent on the EGFR dimerization, on the phosphorylation of tyrosine 845 of EGFR and on the PI3K activity.

3.3.2. Co-localization of EGFR and PKCζ

We performed co-localization studies with green fluorescent protein fusion variants of both proteins as an additional approach to study the association of EGFR and PKCζ. First we proved that PKCζ fused to either EYFP or ECFP is also tyrosine phosphorylated in response to EGF stimulation (Figure 3.16.A). Figure 3.16.B demonstrates that EGF stimulation of EGFR-CFP-expressing cells leads also to tyrosine phosphorylation of PKCζ. In both cases the intensity of PKCζ tyrosine phosphorylation is similar to that of wild type PKCζ upon stimulation of wild type EGFR. We selected Lipofetamine 2000 transfection for the colocalization studies as less toxic for the cells and confering equal delivery of coexpressed proteins. COS-7 cells were transfected with EGFR-CFP and PKCζ-EYFP alone or in combination. The cellular localization of fluorescent label-fused EGFR and PKCζ was detected by the use of laser scanning microscopy on fixed cells.

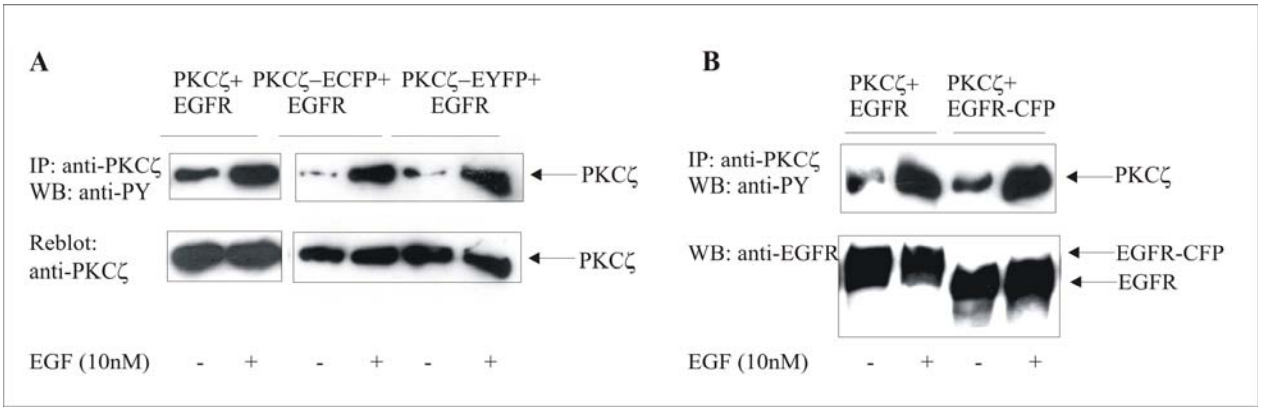


Figure 3.16. Characterization of EGFR-CFP and PKCζ-EYFP/ECFP fusion constructs. COS-7 cells were cotransfected by DEAE-dextran method with 100ng/10^5 cells PKCζ, PKCζ-EYFP or PKCζ-ECFP along with 100ng/10^5 cells EGFR (A) or with 40ng/10^5 cells PKCζ along with the same amount of either EGFR or EGFR-CFP (B). PKCζ immunoprecipitates were analysed with phosphotyrosine-specific antibody and reprobed with PKCζ-specific antibody. Similar results were obtained in three independent experiments.

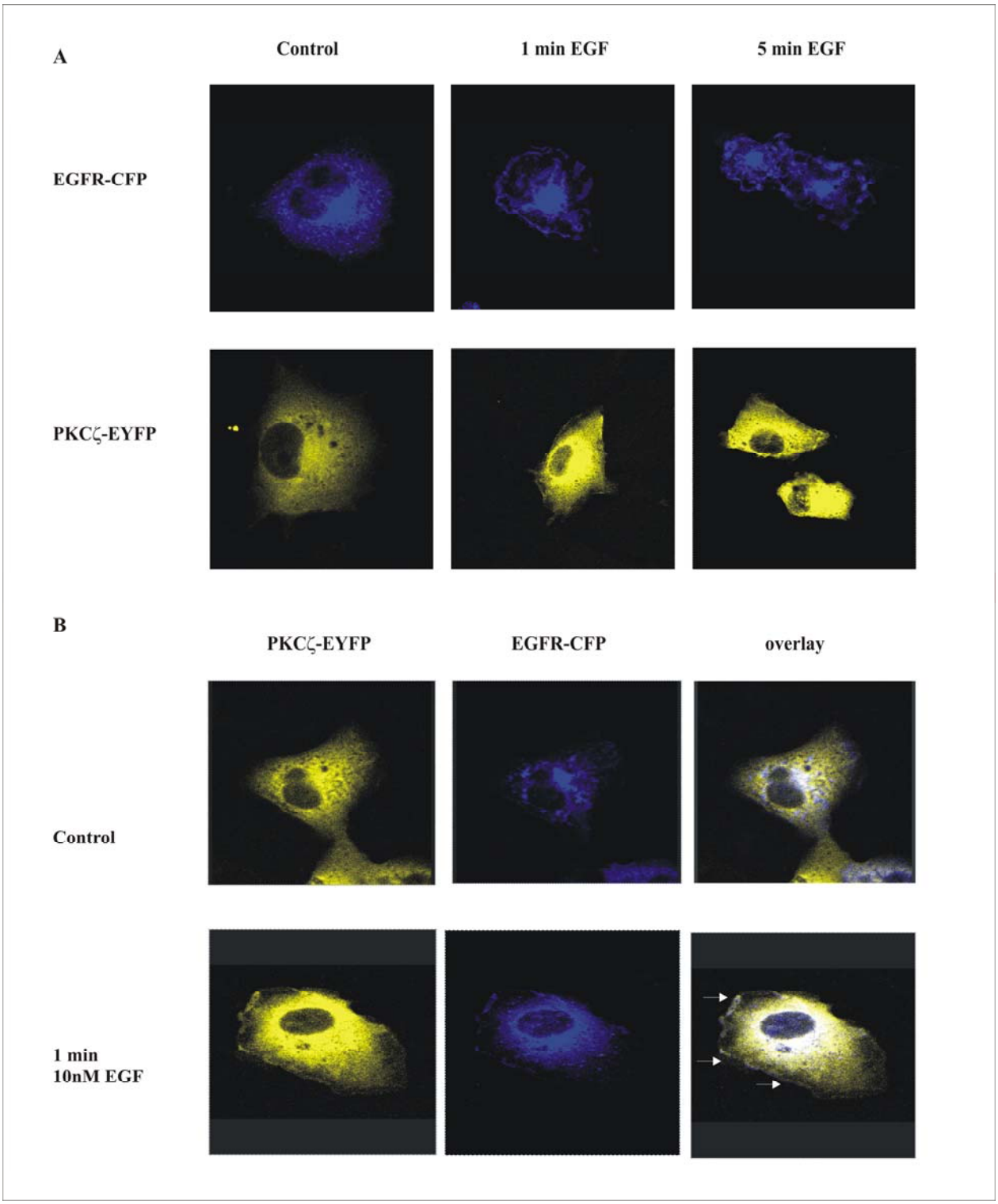


Figure 3.17. Co-localization of EGFR-CFP and PKCζ-EYFP. COS-7 cells were transfected in 6 well plates using Lipofectamine 2000 with either 2.5μg EGFR-CFP or 1.5μg PKCζ-EYFP (A) or co-transfected with 2.5μg EGFR-CFP along with 1.5μg PKCζ-EYFP (B). Serum-starved cells either left untreated or stimulated with 10nM EGF for 1 min or 5 min (A) or for 1 min (B) were fixed. Confocal fluorescent images of CFP, YFP ant their overlay were obtained using laser scanning microscope. The arrows (B) point to the colocalized structures in the overlay.

Figure 3.17.A shows the distribution of EGFR-CFP and PKCζ-EYFP upon their single overexpression. Figure 3.17.B demonstrates the subcellular localization of EGFR-CFP and PKCζ-EYFP in cells cotransfected with both expression constructs. Under unstimulated conditions PKCζ label was diffusely distributed throughout the whole cytoplasm with some concentration in the perinuclear area. Upon stimulation with 10nM EGF for 1min there is translocation of PKCζ to the plasma membrane. Membrane localization of PKCζ was reduced after 5 min EGF simulation. EGFR showed cytoplasmic granular localization under basal conditions without significant membrane labeling what is consistent with the basal localization reported by Yamazaki *et al*., 2002. In response to 1 min EGF stimulation significant EGFR label is found on the membrane and after 5 min EGF stimulation there was increasingly ruffled appearance of EGFR label on the plasma membrane (Figure 3.17.). In fact, only a small part of both EGFR and PKCζ translocates to the membrane, probably due to their high overexpression level. Co-expression of EGFR did not change the subcellular localization of PKCζ. In contrast EGFR subcellular localization is changed upon PKCζ co-expression with more granular cytoplasmic and perinuclear localization under basal conditions and less membrane labeling in response to EGF stimulation. There was some co-localization of EGFR and PKCζ without EGF stimulation in the perinuclear region. Upon stimulation with 10nM EGF for 1 min EGFR as well as PKCζ are detected on the plasma membrane and the overlay indicated that both molecules colocalize at the cell surface (Figure 3.17.B). Thus, PKCζ translocation to the membrane and co-localization with EGFR in response to EGF stimulation observed by laser scanning microscopy represent an additional evidence for their interaction.

3.4. Identification of tyrosine 417 of PKCζ as phosphorylation site

Next question we addressed was which are the tyrosine residues of PKCζ targeted by EGFR-induced phosphorylation. We used site-directed mutagenesis in order to introduce point mutation in PKCζ coding sequence, conferring substitution of particular tyrosine residue with phenylalanine, amino acid that is structurally related to the tyrosine but unphosphorylatable one. The vector construct with mutated PKCζ cDNA was used for transient transfection in COS-7 cells for overexpression of mutated PKCζ protein, harboring the respective amino acid substitution. The residue we selected for mutagenesis was the highly conserved among all PKC isoforms as well as in the whole ABC family of protein kinases tyrosine neighboring the threonine residue at the activation loop. This conserved tyrosine resides in highly conserved region of the PKC catalytic domain (Figure 3.18.A).

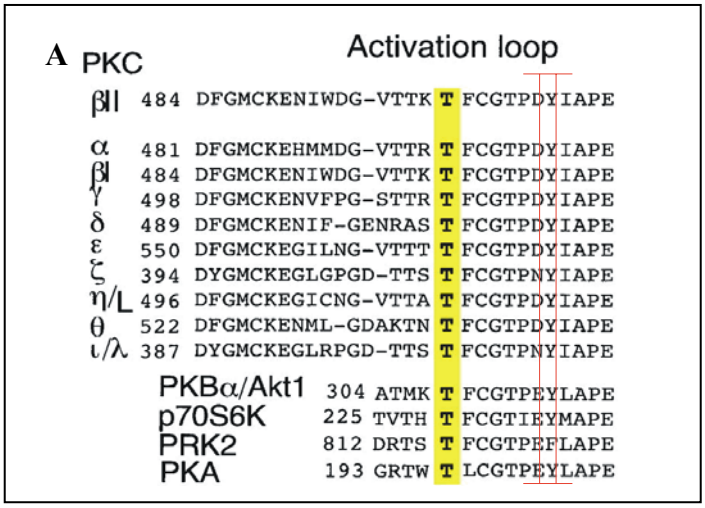
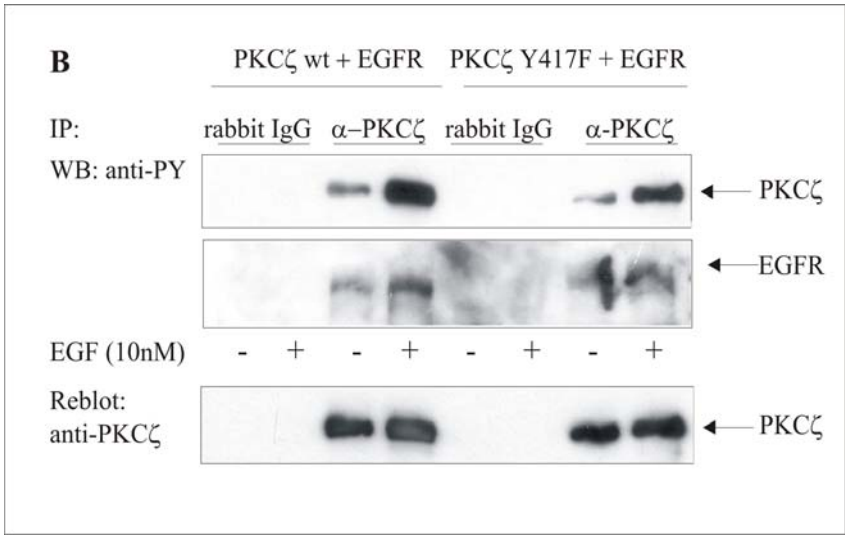


Figure 3.18. EGF-induced phosphorylation of PKCζ triggers tyrosine 417.
(A) Protein kinase C activation loop harbors conserved threonine and conserved tyrosine residue. Present is an alignment of the sequences surrounding activation loop threonine between PKC family members, Akt1, p70S6k, PRK2 and PKA. The activation loop threonine residue is labeled in yellow; the neighboring tyrosine is marked with red lines.



(B) COS-7 cells were cotransfected with 100ng/10^5 cells EGFR along with 100ng/10^5 cells of either wild type PKCζ or PKCζ Y417F. Serum-starved cells were treated with 10nM EGF as indicated. Immunoprecipitation was carried out either with PKCζ-specific antibody or the same amount of normal rabbit IgG as negative control. Immunoprecipitates were analysed by Western blotting with phosphotyrosine-specific antibody PY99 and reprobed with PKCζ- specific antibody.

Comparison of the EGFR-induced tyrosine phosphorylation of PKCζ Y417F to that of wild type PKCζ in COS-7 cells cotransfected with EGFR revealed that its basal tyrosine phosphorylation is reduced compared to that of the wild type PKCζ. The EGF-induced tyrosine phosphorylation of PKCζ Y417F is reduced despite that the mutant is obviously still targeted by tyrosine phosphorylation (Figure 3.18.B). This revealed that Y417 is one of the tyrosines phosphorylated downstream of EGFR stimulation but that the EGFR-mediated modification targets other tyrosines as well. The interaction of Y417F PKCζ with the EGFR was not affected indicating that the association of PKCζ with the receptor is not dependent on the phosphorylation of this particular tyrosine residue.

3.5. EGFR-induced phosphorylation of PKCζ on tyrosine 417 is critically implicated in PKCζ activation downstream of EGFR stimulation

In order to estimate the influence of EGFR stimulation-induced tyrosine phosphorylation on PKCζ enzyme activity we performed kinase activity assay of immunoprecipitated PKCζ with MBP (myelin basic protein) as phosphorylation substrate. Despite that after long exposure period we detected activation of endogenous PKCζ upon EGFR stimulation with 10nM EGF for 5 min (Figure 3.19.A), completely endogenous system did not allow us to study PKCζ activity in relation to its tyrosine phosphorylation. Overexpressed by transient transfection PKCζ had significantly elevated basal activity compared to the endogenous PKCζ activity (Figure 3.19.B).

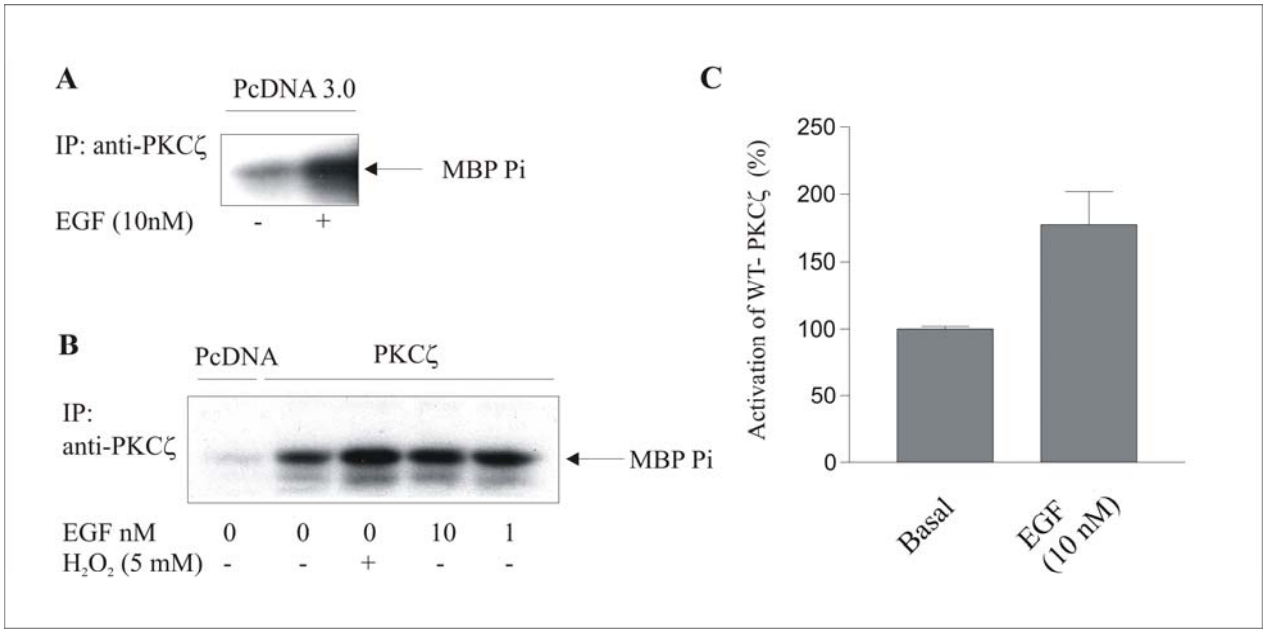


Figure 3.19. PKCζ activation by EGF stimulation. COS-7 cells were transfected with empty vector (A and B) or with PKCζ 150ng/10^5 cells (B). Serum-starved cells were left untreated or treated either with 5 mM hydrogen peroxide for 10 min (B) or with EGF as indicated. PKCζ was immunoprecipitated and kinase assay was carried out with myelin basic protein as substrate. (C) EGF-induced PKCζ activation in relation to basal PKCζ activity. Resuts are expressed as means ± standart errors from five independent experiments.

The high basal activity of PKCζ is known to cause difficulties in detecting stimulus-dependent activation but we found that overexpressed PKCζ is activated upon EGFR stimulation with 1-10nM EGF for 5 min. Hydrogen peroxide treatment was used as a positive control for PKC activation in COS-7 cells (Konishi *et al*., 1997). PKCζ activity in response to 10nM EGF stimulation was quantified as 180% compared to the basal PKCζ activity (Figure 3.19.C). Moreover the PKCζ activity was corresponding to its tyrosine phosphorylation state. Inhibitors that influenced the tyrosine phosphorylation of PKCζ affected its activity state as well. The EGF-dependent increase in PKCζ activity was sensitive to pretreatment with AG1478, thereby being

dependent on EGFR tyrosine kinase activity (Figure 3.20.). Despite of its partial effect on the tyrosine phosphorylation pretreatment with PI3K inhibitor LY294002 also abolished EGF-induced increase in PKCζ activity, demonstrating dependence on PI3K activity during PKCζ activation downstream of EGFR stimulation. Interestingly, both EGF and H_2O_2 treatments further stimulated the elevated basal activity of constitutive active mutant of PKCζ in parallel with increase in its tyrosine phosphorylation. Moreover, the EGF-dependent increase in the activity of constitutive active PKCζ was AG1478 sensitive like that of wild type PKCζ (data not shown).

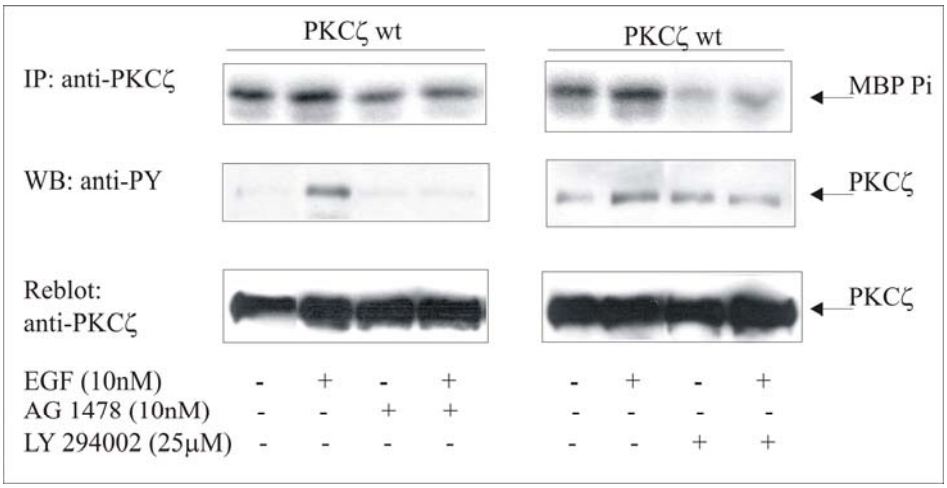


Figure 3.20. EGFR and PI3K are involved in EGF-induced PKCζ activation. Serum-starved COS-7 cells transfected with 150ng/10^5 cells PKCζ were pretreated either with 10nM AG1478 or with 25 μM LY294002 for 30 min prior to stimulation with 10nM EGF for 5 min as indicated. PKCζ was immunoprecipitated and immune complex kinase assay with MBP as substrate was performed. PKCζ immunoprecipitates used in the kinase assay were analysed by Western blotting with phosphotyrosine-specific antibody PY99 and reprobed with PKCζ-specific antibody. Similar results were obtained in three independent experiments.

Next step was to estimate whether the phosphorylation of PKCζ on tyrosine 417 contributes to its EGFR-dependent activation. Surprisingly the mutant behaved as inactive one. The basal activity of PKCζ Y417F was significantly reduced and only weak activation was detected upon H_2O_2 treatment whereas EGF treatment did not influence its activity at all (Figure 3.21.). In addition to MBP phosphorylation PKCζ autophosphorylation was estimated. Both H_2O_2 and EGF stimulated the autophosphorylation of wild type PKCζ with stronger effect of H_2O_2. Similar to the pronounced decrease in MBP phosphorylation by PKCζ Y417F mutant, its autophosphorylation is also affected and slightly stimulated only by H_2O_2 but not influenced by EGF. Again a correlation between the activity and the tyrosine phosphorylation was identified with higher phosphorylation and respectively higher activation induced by H_2O_2. The remaining tyrosine phosphorylation, which EGF stimulation induces on PKCζ Y417F obviously did not contribute to PKCζ activation. For comparison H_2O_2-induced tyrosine phosphorylation of PKCζ Y417F was

not significantly different to that of the wild type but also was not related to pronounced PKCζ activation.

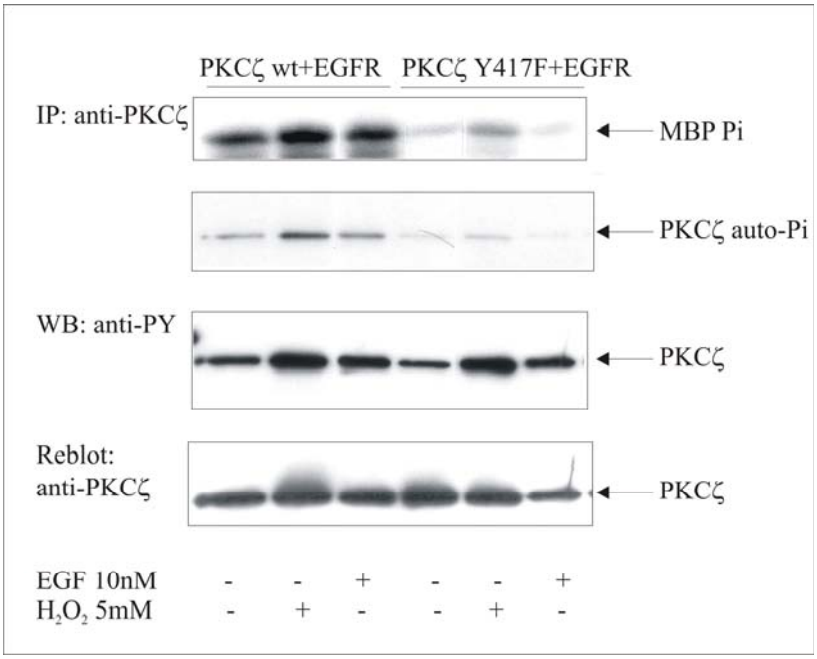


Figure 3.21. Comparison of PKCζ wt and PKCζ Y417F activity upon EGF and hydrogen peroxide stimulation. Serum-starved COS-7 cells cotransfected with 100ng/10^5 cells EGFR along with 100ng/10^5 cells either wild type PKCζ or PKCζ Y417F were stimulated with 5mM hydrogen peroxide for 5 min or 10 nM EGF for 5 min as indicated. PKCζ was immunoprecipitated and immune complex kinase assay with MBP as substrate was performed. In addition to MBP phosphoryaltion PKCζ autophosphorylation was estimated. PKCζ immunoprecipitates used in the kinase assay were analysed by Western blotting with phosphotyrosine-specific antibody PY99 and reprobed with PKCζ-specific antibody. Shown is representative of two independent experiments.

We compared the PKCζ mutant harboring substitution of the activation loop threonine to alanine (T410A) and PKCζ Y417F concerning their tyrosine phosphorylation and activity state upon EGFR stimulation. As it was expected on the basis of well-established critical role of the threonine phosphorylation at the activation loop for PKCζ activity (Chou *et al*., 1998), PKCζ T410A was inactive, with low basal activity and absence of activation by EGFR stimulation. However, PKCζ T410A is tyrosine phosphorylated in response to EGFR stimulation. PKCζ Y417F basal activity and the activity upon EGF stimulation did not differ significantly from that of PKCζ T410A (Figure 3.22.). EGF stimulates the autophosphorylation of wild type PKCζ. PKCζ T410A had lowered and non-stimulated by EGF autophosphorylation, whereas the autophosphorylation of PKCζ Y417F was slightly higher but still unresponsive to EGF stimulation. Site-directed mutagenesis procedure targeting tyrosine-417 did not change the threonine-410 and respectively threonine-410 mutagenesis did not alter tyrosine-417. Thus, it appeared the tyrosine phosphorylation could not induce activation in the absence of threonine phosphorylation at the activation loop. Vice versa PKCζ Y417F harbors intact activation loop threonine but nevertheless is inactive. Therefore both the basal activity of PKCζ and EGFR-

dependent activation of PKCζ require not only phosphorylation of the threonine at the activation loop, but phopshorylation of the neighboring tyrosine residue as well.

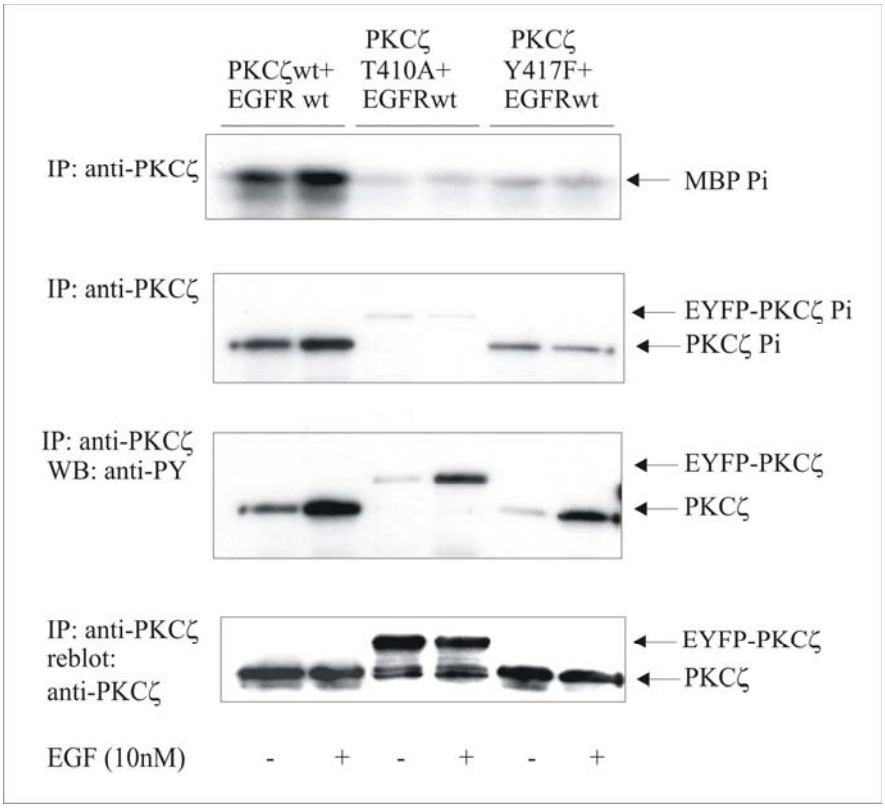


Figure 3.22. Activity of wild type PKCζ, PKCζ Y417F and PKCζ T410A upon EGF stimulation. Serum-starved COS-7 cells were cotransfected with 100ng/10^5 cells EGFR along with 100ng/10^5 cells either wild type PKCζ, PKCζ T410A-EYFP or PKCζ Y417F were stimulated with 10nM EGF as indicated. PKCζ was immunoprecipitated ánd immune complex kinase assay with MBP as substrate was performed. PKCζ autophosphorylation was estimated as well. PKCζ immunoprecipitates used in the kinase assay were analysed by Western blotting with phosphotyrosine-specific antibody PY99 and reprobed with PKCζ-specific antibody. Shown is representative of three independent experiments.

3.6. Role of PKCζ and its tyrosine phosphorylation in the EGFR-activated signalling pathways

3.6.1. MAPK (Erk1/Erk2) activation

PKCζ is involved in the MAPK signalling cascades in many cell types and in the EGFR-induced Erk1/2 activation as well (Hirai and Chida, 2003). We determined the effect of PKCζ overexpression on the EGF-induced Erk activation by MBP phosphorylation assay with immunoprecipitates of overexpressed HA-MAPK (Erk2) along with immunoblot analysis with phospho-specific antibody (anti-phospho-Erk1/2). Moreover we compared the influences of wild type PKCζ, PKCζ Y417F and PKCζ T410A mutants on EGF-induced MAPK activation.

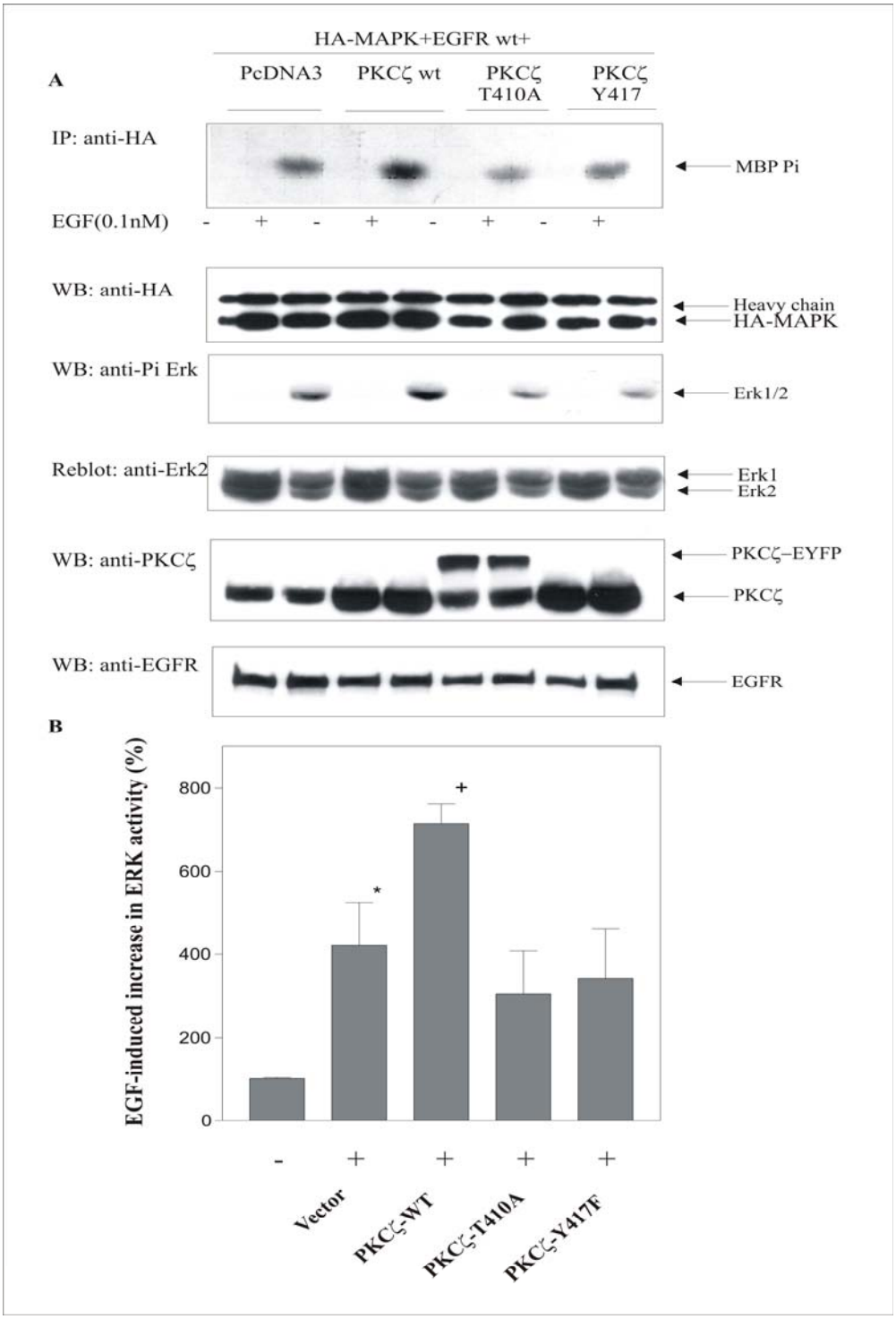


Figure 3.23. Influence of wt PKCζ, PKCζ Y417F and PKCζ T410A on activation of MAPK. (A) COS-7 cells were cotransfected with 70ng/10^5 cells HA-MAPK along with 40 ng/10^5 cells EGFR and 40 ng/10^5 cells of either empty vector, wild type PKCζ, PKCζ T410A-EYFP or PKCζ Y417F. Serum-starved cells were stimulated with 0.1nM EGF for 5 min as indicated. Activity assay was performed on MAPK immunoprecipitated with anti-HA antibody using MBP as substrate. MBP phosphorylation was detected by autoradiography. Immunoprecipitates were analysed by Western blotting with HA tag-specific antibody. In parallel aliquots (corresponding to 10^5 cells) of the same lysates analysed by Western blotting with phospho-Erk1/2-specific antibody, EGFR-specific antibody 13G8 or PKCζ-specific antibody and reprobed Erk2-specific antibody. EGF-induced increase in ERK activity expressed in percentage related to the basal activity upon expression of wt PKCζ, PKCζ Y417F and PKCζ T410A is presented as means ± SEM from four independent experiments (B). *-Significantly different from the basal level, +- significantly higher than the empty vector transfected cells.

Since stimulation with 10nM EGF led to almost maximal MAPK activation no big differences could be seen in the MAPK activity due to the overexpression of PKCζ and/or EGFR (not shown). In contrast, upon cell stimulation with lower EGF concentration (0.1nM) a substantially increased EGF-induced MAPK activation was detected in cells that overexpress wild type PKCζ (Figure 3.23.A). The quantification shown on Figure 3.23.B. revealed that EGF stimulates MAPK activity in empty vector-transfected cells around four-fold and that EGF-stimulated MAPK activity increased 1.75 fold upon overexpression of wild type PKCζ. Upon both PKCζ T410A and PKCζ Y417F overexpression the effect of PKCζ overexpression on MAPK activity downstream of EGFR is abolished and EGF-induced MAPK activity is similar to that in empty vector-transfected cells. The same trend was detected when instead of radioactive HA-MAPK activity assay Western blot analysis with phospho-Erk1/2-specific antibody was performed. Thus PKCζ catalytic activation in response to EGFR stimulation contributes to the MAPK (Erk1/Erk2) activation downstream of EGFR. Since both catalytically inactive mutants are unable to exert such an effect, the PKCζ catalytic activation and EGF-induced tyrosine phosphorylation as its integral part are critically involved in EGFR-induced MAPK pathway.

3.6.2. Akt/PKB pathway

Next we assessed the role of PKCζ in Akt/PKB activation downstream of EGFR stimulation. We analysed Akt activation using phospho-specific antibody that recognizes the indicative for activation phosphoserine 473. Akt phosphorylation/activation reached maximum after 5-10 min EGF stimulation and gradually decrease upon longer EGF treatment (not shown). We estimated the impact of PKCζ overexpression or coexpression with EGFR on activation-related Akt phosphorylation upon stimulation with lower EGF concentrations (0.1-1 nM). As it is shown on Figure 3.24.A the overexpression of wild type PKCζ did not affect significantly EGF-induced Akt phosphorylation. In cells transfected either with empty vector, EGFR or wild type PKCζ EGF-induced Akt phosphorylation is higher compared to the cells cotransfected with PKCζ and EGFR. Thus Akt activation is counteracted in cells overexpressing both EGFR and PKCζ. When instead of wild type PKCζ PKCζ Y417F was transfected along with EGFR, Akt phosphorylation upon stimulation with 0.1-1nM EGF was significantly less. Therefore, it seems that phosphotyrosine 417 of PKCζ is implicated in Akt phosphorylation and activation in EGFR downstream signalling. Since overexpression of wild type PKCζ does not influences positively Akt phosphorylation it might be that the phosphorylation of tyrosine 417 itself, rather than the catalytic activity of PKCζ contributes to Akt phosphorylation and activation.

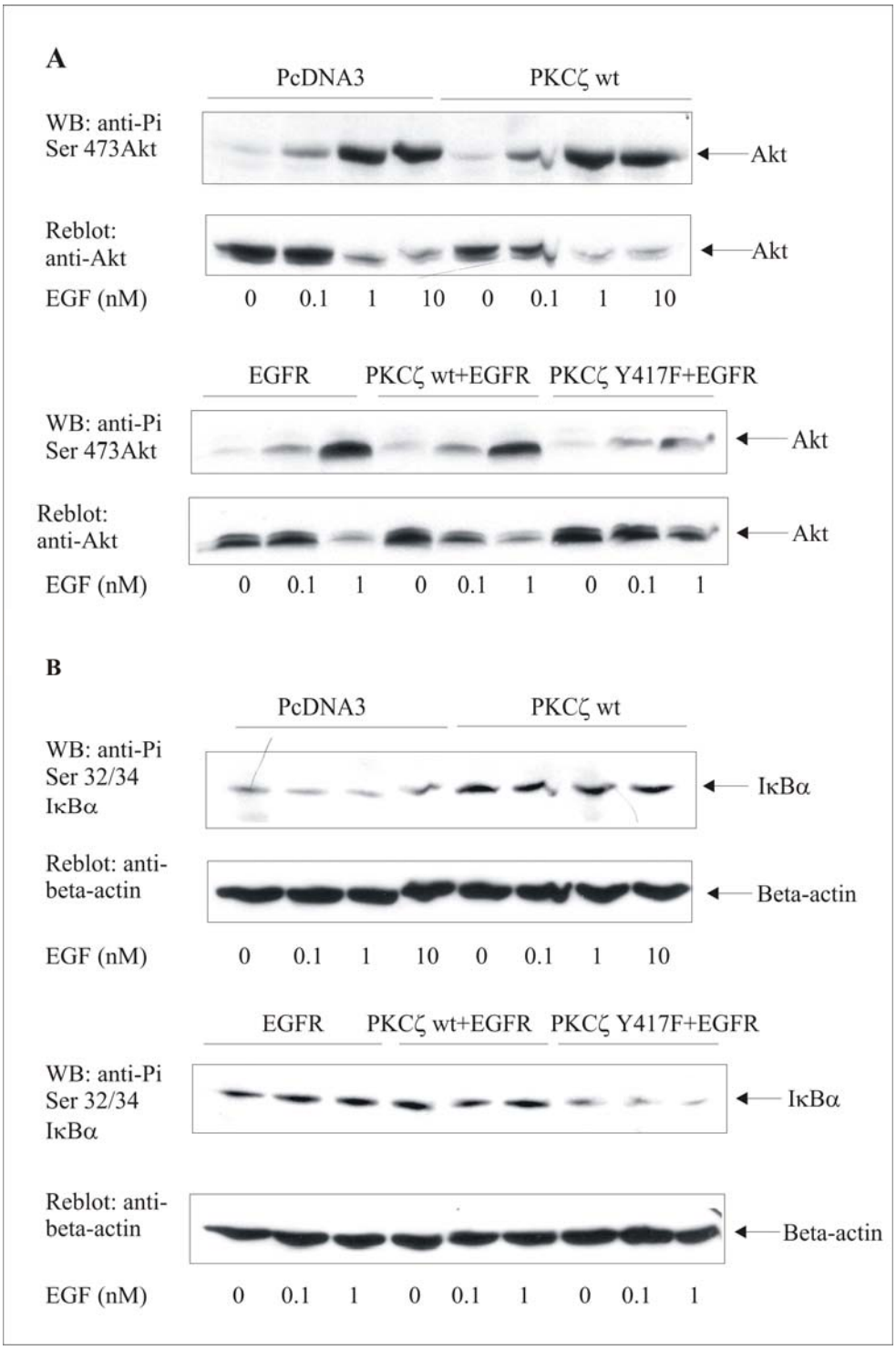


Figure 3.24. Influence of PKCζ and PKCζ Y417F on Akt activation (A) and on IκBα phosphorylation downstream of EGFR (B). COS-7 cells were transfected with empty vector, 40ng/10^5 cells EGFR or 40ng/10^5 cells PKCζ or co-transfected with EGFR and either wild type PKCζ or PKCζ Y417F. Serum-starved cells were stimulated with 0.1-10 nM EGF for 5 min as indicated. Cell lysates from 10^5 cells were analysed by Western blotting with phospho-Akt (Ser473)-specific antibody (A) or with anti-phospho- (Ser 32/34) IκBα antibody (B) and respectively reprobed with Akt-specific antibody (A) or with beta-actin-specific antibody as a control for equal protein loading. Results are representative of two independent experiments.

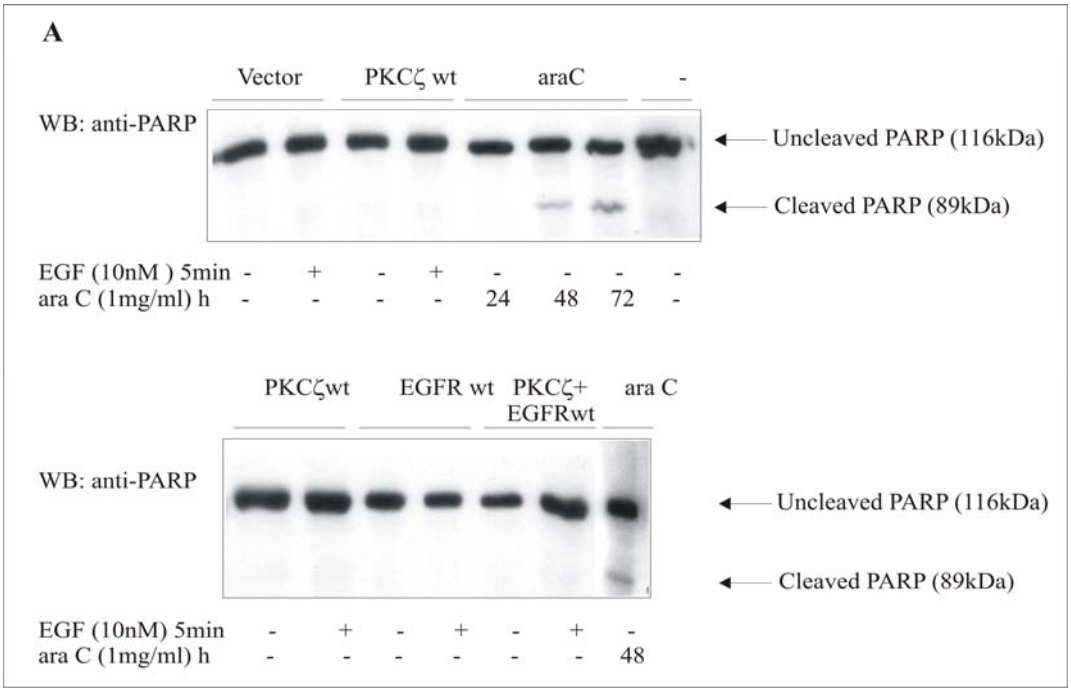
3.6.3. NFκB pathway-IκBα phosphorylation

IκBα phosphorylation increased upon stimulation of COS-7 cells with 10nM EGF as detected by phospho- IκBα (Ser32/36) monoclonal antibody. This was indicative for NFκB activation downstream of EGFR receptor activation in cells transfected with either empty vector, PKCζ or EGFR (Figure 3.24.B and not shown) Cells transfected with PKCζ, EGFR, or both together showed elevated basal phosphorylation of IκBα compared to empty vector-transfected cells thus indicating for involvement of both EGFR and PKCζ in signalling leading to phosphorylation of IκBα. Overexpression of PKCζ Y417F instead of wild type PKCζ caused decreased IκBα phosphorylation, demonstrating in this way role for PKCζ activity itself or for tyrosine-417 phosphorylation in IκBα phosphorylation. Therefore, it seems that catalytic activation of PKCζ as well as its phosphorylation on tyrosine 417 upon EGFR stimulation are implicated in many aspects of the proliferative and pro-survival signalling downstream of EGFR.

3.7. Role of PKCζ in the cell signalling – towards apoptosis or cell growth?

In the literature there are reports about pro-apoptotic as well as for pro-survival role of PKCζ. We checked whether PKCζ influences either the rate of cell apoptosis or the proliferating capabilities of COS-7 cells. Figure 3.25.A shows the absence of cleavage of poly- (ADP-ribose)-polymerase (PARP)- an early characteristic marker for cells undergoing apoptosis under conditions and combinations we used for transient transfection of COS-7 cells. Cleaved fragment of PARP was detected only in cells treated for 48-72 h with araC (cytarabin, 1βD-arabinofuranosylcytosine) as positive control for PARP cleavage induction. We carried out [^{3}H]-thymidine incorporation assay to estimate the influence of PKCζ on cell proliferation. It was problematic to perform such proliferation assay with COS-7 cells transfected with DEAE-dextran transfection method. Obviously due to the elevated toxic effect during the transfection procedure, we obtained only a small proliferative response to stimulation with 10nM EGF. Therefore we used Polyfect as another transfection method, which is less toxic for the cells. 10nM EGF stimulation of empty vector transfected cells led to more that 200% increase in the level of [^{3}H]-thymidine incorporation (Figure 3.25.B). Overexpression of wild type PKCζ induced increased DNA synthesis in the absence of any stimulation as measured by [^{3}H]-thymidine incorporation. PKCζ overexpressing cells showed the same level of DNA synthesis upon 10nM EGF stimulation as empty vector-transfected ones. Upon transfection of PKCζ Y417F the basal DNA synthesis was reduced but still significantly elevated in comparison to the wild type PKCζ effect. However, the incorporation of [^{3}H]-thymidine in response to 10nM EGF stimulation was clearly reduced in cells transfected with PKCζ Y417F mutant. These results demonstrate that PKCζ has a mitogenic

effect, which is lost in PKCζ Y417F mutant, either due to its catalytic inactivity, or to the absence of tyrosine phosphorylation on tyrosine 417.



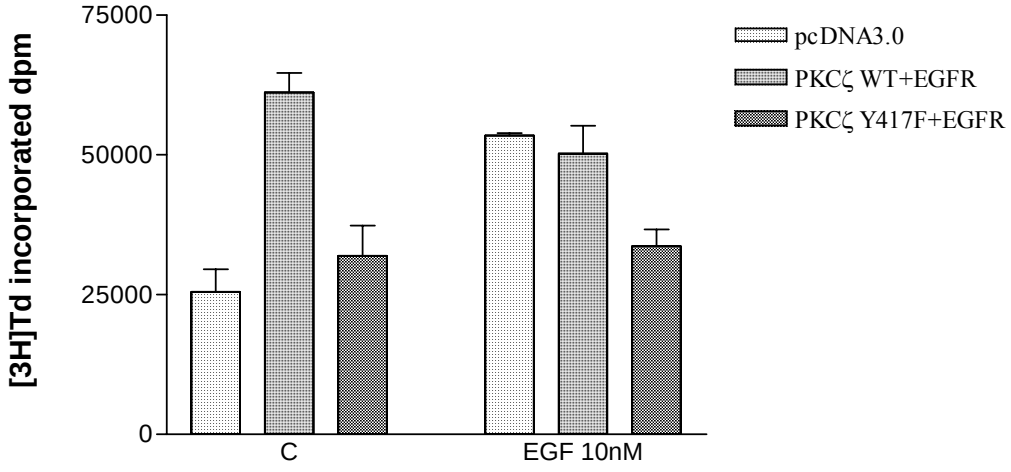


Figure 3.25. Effect of PKCζ on apoptosis and cell growth. (A) COS-7 cells transfected with empty vector, 100 ng/10^5 cells EGFR, 100ng/10^5 cells PKCζ or cotransfected with EGFR and PKCζ were serum-starved and stimulated with 10nM EGF as indicated. Whole cell lysates were prepared and analysed by Western blotting with PARP-specific antibody, which recognizes full length PARP as well as the large PARP fragment generated by caspases. Negative control was lysate from non-transfected COS-7 cells, indicated with (-). Non-transfected COS-7 cells were treated for indicated time periods with 1mg/ml araC as positive control. Arrows point to the positions of full length PARP-116kDa and the large product of cleavage-89kDa. (B) Effect of EGF stimulation on the DNA synthesis versus the basal level upon transfection with empty vector, wild type PKCζ or PKCζ Y417F. COS-7 cells were transfected in 24-well plates with empty vector, 100ng EGFR together with 100ng either wild type PKCζ or PKCζ Y417F using Polyfect transfection method. Serum-starved cells were stimulated with 10nM EGF for 20 hours and [^{3}H]-thymidine incorporation assay was carried out. Results expressed as means ± SEM from two independent experiments in triplicate.

3.8. Tyrosine phosphorylation of PKCζ induced by stimulation of G-protein coupled receptors

Next question we asked was whether stimulation of G-protein coupled receptors could also induce tyrosine phosphorylation of PKCζ. We estimated PKCζ tyrosine phosphorylation upon stimulation of the endogenously expressed in COS-7 cells GPCRs -lysophosphatidic acid receptor and beta 2-adrenergic receptor with lysophosphatidic acid (LPA) and Isoproterenol (Iso), respectively. For both receptors signalling pathways involving EGFR transactivation and downstream MAPK/Erk activation are reported (Daub *et al*., 1997, Maudsley *et al*., 2000). First we compared the EGF, LPA (A) and Iso (B)-induced tyrosine phosphorylation of PKCζ under different overexpression conditions (Figure 3.26). In COS-7 cells transfected only with empty vector it was not possible to detect EGF or LPA-induced tyrosine phopshorylation of PKCζ, whereas stimulation with Iso led to slight increase in the tyrosine phosphorylation of endogenous PKCζ. In cells transfected with PKCζ alone the increased amount of PKCζ allowed detection of its tyrosine phosphorylation under basal conditions. Although PKCζ tyrosine phosphorylation does not change significantly upon 1 nM EGF stimulation, it increased slightly upon stimulation with both 10μM LPA and 10μM Iso. In cells co-transfected with PKCζ and EGFR the basal as well as stimuli-induced tyrosine phosphorylation of PKCζ were elevated. Clearly both LPA and Iso stimulations induced PKCζ tyrosine phosphorylation similar to that induced by 1nM EGF stimulation. Moreover, EGFR co-immunoprecipitation with PKCζ was identified upon LPA and Iso stimulation like upon EGF stimulation. The phosphotyrosine band at the level of EGFR was visible in PKCζ immunoprecipitates upon EGF and Iso stimulation under completely endogenous conditions as well as under basal, EGF, LPA or Iso stimulated conditions in cells overexpressing PKCζ alone. In cells co-transfected with PKCζ and EGFR there was pronounced increase in the tyrosine phosphorylation of EGFR co-immunoprecipitating with PKCζ upon EGF, LPA and Iso treatment. Both LPA and Iso stimulation mediated ERK and LPA mediates Akt activation as well. LPA and Iso-mediated ERK activation was lowered in double overexpression system, whereas LPA-mediated Akt activation was not changed. Next step was to determine whether GPCR-mediated tyrosine phosphorylation of PKCζ target also tyrosine-417 in the activation loop vicinity. On Figure 3.27.A is presented a comparison of EGF, LPA and Iso-induced tyrosine phosphorylation of wild type PKCζ and of PKCζ Y417F mutant. EGF-induced tyrosine phosphorylation of PKCζ Y417F is normally only partly decreased (as shown on Figure 3.18. B).

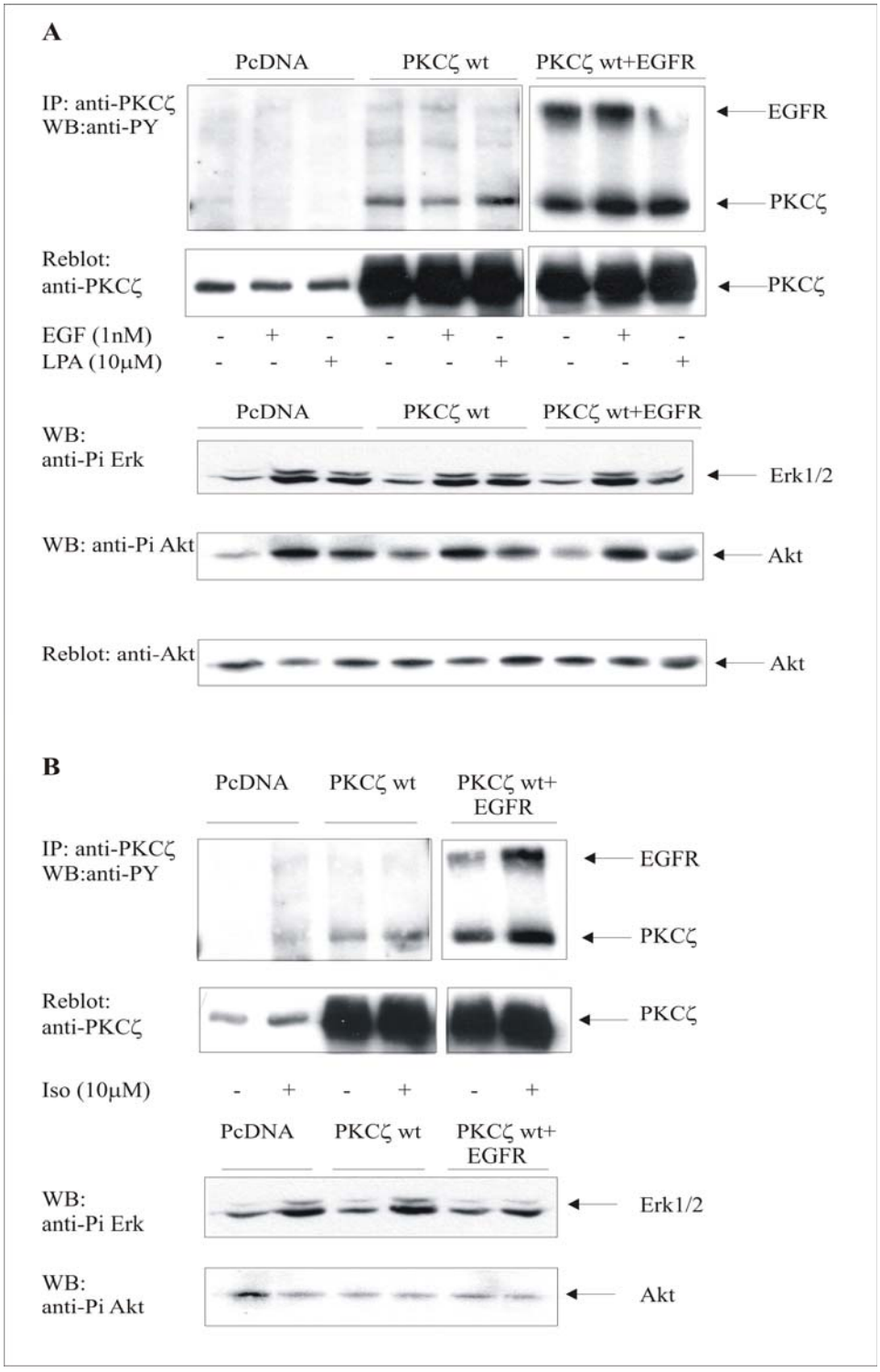


Figure 3.26. LPA and Iso stimulation also induce tyrosine phosphorylation of PKCζ. COS-7 cells were transfected with either empty vector pcDNA 3.0, 70ng/10^5 cells PKCζ or 70ng/10^5 cells PKCζ along with 40ng/10^5 cells EGFR. Serum-starved cells were left untreated or stimulated with 1nM EGF, 10μM LPA (A) or 10μM Iso (B) for 5 min. PKCζ immunoprecipitates were by Western blotting with phosphotyrosine-specific antibody 4G10, and reprobed with PKCζ-specific antibody. Cell lysates (corresponding to 10^5 cells) were analysed by western blotting with phospho-Erk1/2 or phospho-Akt-specific antibodies and reprobed with anti-Akt antibody. Similar results were obtained in two independent experiments.

The stronger decrease in the experiment shown on Figure 3.27.A was due to the lower amount immunoprecipitated PKCζ on the respective lane. LPA stimulation seems to be not able to induce tyrosine phosphorylation of PKCζ Y417F indicating that tyrosine-417 is the main target of LPA receptor-mediated PKCζ tyrosine phosphorylation. PKCζ Y417F was only faintly tyrosine phosphorylated upon Iso stimulation, suggesting that for beta 2-adrenergic receptor-mediated PKCζ tyrosine phosphorylation tyrosine–417 is a major target as well. It is well known that transactivation of EGFR could be mediated via release of HB-EGF (Prenzel *et al*., 1999). Therefore we asked whether HB-EGF stimulation could also induce tyrosine phosphorylation of PKCζ. Figure 3.27.B shows that HB-EGF is indeed able to induce tyrosine phosphorylation of PKCζ. EGF-induced tyrosine phosphorylation of PKCζ was sensitive to inhibition of EGFR tyrosine kinase activity by AG1478.

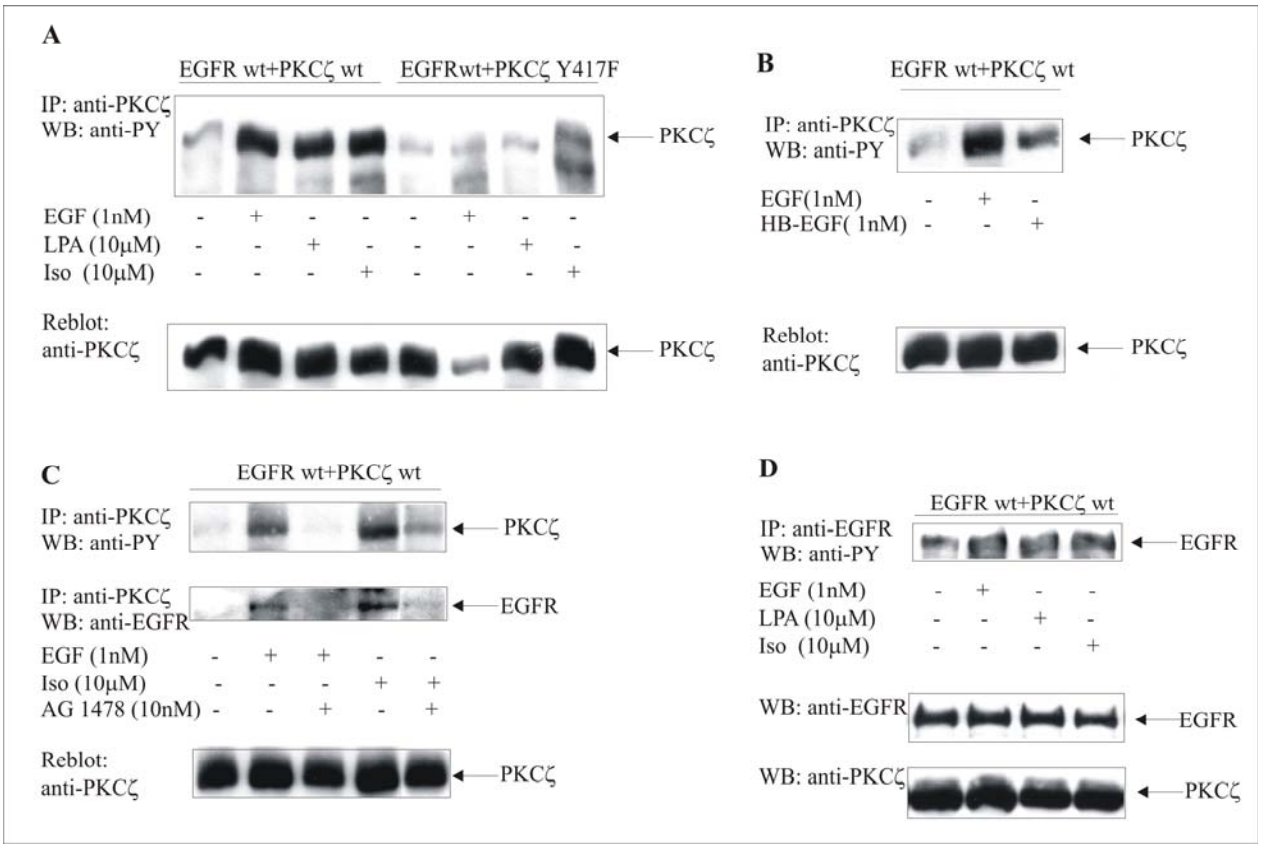


Figure 3.27. LPA and Iso–induced tyrosine phosphorylation of PKCζ triggers tyrosine 417 as main target via transactivation of EGFR. COS-7 cells were cotransfected with 40ng/10^5 cells EGFR and 40ng/10^5 cells either wild type PKCζ (A, B, C, D) or PKCζ Y417F (A). Serum-starved cells were preincubated with AG1478 for 30 min (C) and treated with 1nM EGF, 1nM HB-EGF, 10μM LPA or 10μM Iso for 5 min as indicated. Either PKCζ (A, B and C) or EGFR (D) was imunoprecipitated and analysed by Western blotting with phosphotyrosine-specific antibody 4G10 (A, B, C and D) and reprobed with PKCζ-specific antibody (A, B and C). Cell lysates (corresponding to 10^5 cells) were analysed by western blotting with EGFR-specific antibody 13G8 and PKCζ-specific antibody. Results are representative of three independent experiments.

We identified that at least Iso-induced tyrosine phosphorylation of PKCζ was sensitive in part to AG1478 treatment (Figure 3.27.C). AG1478 treatment influenced in the similar manner the co-immunoprecipitation of EGFR with PKCζ upon EGF and Iso stimulation. In addition we found by Western blot with phosphotyrosine-specific antibody of EGFR immunoprecipitates that both LPA and Iso induced increase in the tyrosine phosphorylation of EGFR (Figure 3.27.D). This is the indication that both GPCRs transduce signals through EGFR transactivation and that transactivated EGFR can also mediate PKCζ tyrosine phophorylation. We performed PKCζ kinase activity assay to evaluate PKCζ activation upon GPCR stimulation. Both LPA and Iso activate wild type PKCζ but do not activate PKCζ Y417F (Figure 3.28).

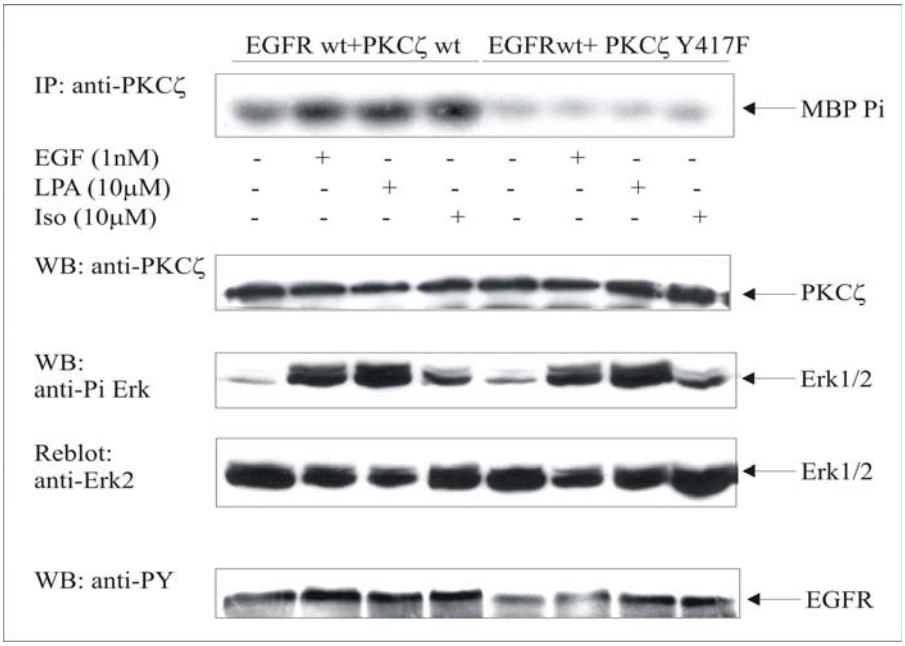


Figure 3.28. GPCR activation by LPA and Iso mediates activation of wild type PKCζ, but not of PKCζ Y417F. COS-7 cells were cotransfected with 40ng/10^5 cells EGFR and 40ng/10^5 cells either wild type PKCζ or PKCζ Y417F. Serum-starved cells were either left untreated or incubated with 1nM EGF, 10μM LPA or 10μM Iso for 5 min as indicated. PKC activity assay was performed on PKCζ immunoprecipitates with MBP as substrate. Immunoprecipitation of equal amount PKCζ was confirmed by immunoblot with PKCζ-specific antibody.). Cell lysates (corresponding to 10^5 cells) were analysed by Western blotting with phospho-Erk1/2-specific antibody or phosphotyrosine-specific antibody and reprobed with Erk2-specific antibody. Shown is a representative of four independent experiments.

This is the indication that the common mechanism for PKCζ activation operates downstream from ligand-activated and transactivated EGFR and that crucial for this activation process is the phosphorylation of tyrosine-417 in the vicinity of PKCζ activation loop.

4. DISCUSSION

In this study we investigated the tyrosine phosphorylation of PKC isoforms overexpressed in COS-7 cells upon EGFR activation. EGFR activation in normal keratinocytes as well as autocrine stimulation of EGFR via TGFα release in ras-transformed keratinocytes has been reported to mediate tyrosine phosphorylation of PKCδ, but not of PKCα, ε, η and ζ (Denning *et al*., 1993, 1996). We found that in COS-7 cells PKCδ but also PKC isoforms ε and ζ are subjected to tyrosine phosphorylation upon EGFR stimulation. The EGF-induced modification of PKCδ, ε and ζ was less pronounced compared to the tyrosine phosphorylation induced by hydrogen peroxide treatment and in the case of PKC δ and ε similar to the effect of phorbol ester treatment (Figure 3.2 and 3.3). Phorbol ester and hydrogen peroxide are well-known inducers of PKC tyrosine phosphorylation. Phorbol ester-induced tyrosine phosphorylation has been reported for PKCα, βII and δ (Braiman *et al*., 1999; Li *et al*., 1994). PKC α, βI, γ, δ, ε and ζ overexpressed in COS-7 cells were tyrosine phosphorylated upon hydrogen peroxide treatment (Konishi *et al*., 1997), independently of the PTP inhibition (Yamamoto *et al*., 2000). In our experiments we observed increased tyrosine phosphorylation of PKCε and ζ in cells co-transfected with EGFR. PKCζ is stronger tyrosine phosphorylated than PKCε upon stimulation of endogenous EGFR whereas in cells co-expressing PKC and EGFR both PKC isoforms were tyrosine phosphorylated to a similar extent (Figure 3.3.). Tyrosine phosphorylation of PKC isoforms downstream of many other receptors has been identified. Activation of FcεRI receptor for IgE in rat basophilic leukemia mast cells RBL-2H3 leads to tyrosine phosphorylation of PKCδ, θ, α and βI (Haleem-Smith *et al*., 1995; Liu *et al*., 2001; Kawakami *et al*., 2003). PKCθ is tyrosine phosphorylated upon activation of adhesion receptors in platelets (Crosby and Poole, 2002) and downstream of TCR in T cells (Liu *et al*., 2000). Stimulation of NGFR mediates tyrosine phosphorylation of atypical PKCs in PC12 cells (Wooten *et al*., 2001a). PKCδ is tyrosine phosphorylated downstream of B cell receptor engagement (Barbazuk and Gold, 1999), insulin receptor stimulation (Rosenweig *et al*., 2004) as well as upon activation of GPCRs by carbachol and substance P (Soltoff *et al*., 1995) and in vitro by PDGFR (Li *et al*., 1994). Braiman *et al*., 1999, have shown that insulin stimulation of primary skeletal muscle cultures induces tyrosine phosphorylation of PKCβII and PKC**ζ.** It is apparent that in most of the cases tyrosine phosphorylation of PKC isoforms is induced by cell surface receptors either with intrinsic tyrosine kinase activity or receptors, which do not possess their own tyrosine kinase activity but recruit cytoplasmic tyrosine kinases upon activation. Even the tyrosine phosphorylation induced by hydrogen peroxide and phorbol ester might be discussed in the same connection, based on the recent findings that H_2O_2 (Fischer *et al*., 2004) as well as PMA (Amos *et al*., 2004) mediate RTK transactivation.

In our experiments the critical involvement of EGFR is supported by the significant increase in PKC tyrosine phosphorylation in cells overexpressing EGFR as well as by its dependence on EGF concentration (Figure 3.4). The time course of the tyrosine phosporylation occurrence revealed that it is very early event upon stimulation of EGFR. It can be detected for both PKCε and ζ to increase rapidly after 30 sec of EGF stimulation and slightly decrease after EGF stimulation for 1 min. Despite of this similarity, there were differences in the time course of the tyrosine phosphorylation of both PKC isoforms upon prolonged EGF stimulation. Whereas PKCζ tyrosine phosphorylation was kept elevated, PKCε tyrosine phosphorylation was gradually increasing in response to EGF stimultion up to 10 min (Figure 3.5.). The different time course of tyrosine phosphorylation of both PKC isoforms could reflect either different kinetic of their phosphorylation or their different susceptibility with PKCζ being more resistant to protein tyrosine phosphatases-mediated PKC dephosphorylation. Indeed, PTPs are also activated by EGFR stimulation and PTPs-dependent dephosphorylation may counteract the tyrosine phosphorylation of PKC. A receptor-tyrosine phosphatase CD45 is shown to be involved in dephosphorylation of PKCδ in monocytic cells (Deszo *et al*., 2001). Haleem-Smith *et al*., 1995 demonstrated that PKCδ tyrosine phosphorylation induced by antigen stimulation of FcεRI has very rapid rate of phosphorylation (detected at 30 sec) followed by rapid dephosphorylation rate since the modification has been no longer detected after 3 min of stimulation. Tyrosine phosphorylation of PKCθ in the same cell system had another time schedule occurring with a delay compared to the rapid modification of PKCδ (Liu *et al*., 2001). We did not assess the involvement of PTPs in the transient decrease in the tyrosine phosphorylation of PKCε and ζ.

EGF-induced tyrosine phosphorylation of PKCζ as well as that of PKCε was completely abolished by pretreatment with EGFR tyrosine kinase inhibitor AG1478, demonstrating the involvement of EGFR tyrosine kinase activity in PKC modification. This finding was further confirmed by the diminishing effect of EGFR dimerization mutant expression on tyrosine phosphorylation of PKCζ (Figure 3.6). Since both AG1478 and EGFR dimerization mutant caused decrease in the basal tyrosine phosphorylation of PKCζ as well, we suggest that overexpressed EGFR evokes PKCζ tyrosine phophorylation under non-stimulated conditions probably due to its basal activity. Thus, it became clear that functional EGFR is required for PKCζ modification by tyrosine phosphorylation. Similarly, tyrosine phosphorylation of PKCδ in keratinocytes induced by TGFα requires a functional EGFR. However, PKCδ was tyrosine phosphorylated *in vitro* by Src and Fyn tyrosine kinases but not by EGFR (Denning *et al*., 1996). Li *et al*., 1994 reported *in vitro* phosphorylation of PKCδ by Fyn, IR and PDGFR. Without to exclude the involvement of receptor tyrosine kinases, many reports from *in vitro* and *in vivo* studies reveal the role for cytoplasmic tyrosine kinases as proximate kinases in PKC tyrosine phosphorylation. Src family kinases - Src, Fyn, Lyn, Lck as well as other cytosolic tyrosine

kinases such as Btk, Abl, Syk, and Ca^{2+}-dependent Pyk2 are implicated in the tyrosine phosphorylation of PKC isoforms in different cell systems upon various stimuli (Table 1). Src family kinases have been implicated in PDGF-induced tyrosine phosphorylation of PKCδ in C6 glioma cells but the authors did not exclude direct phosphorylation of PKCδ by PDGFβR upon PDGF binding (Kronfeld *et al*., 2000). Upon co-expression of wild type Src we observed increased tyrosine phosphorylation of PKCζ, whereas upon transfection of dominant negative Src EGF-induced tyrosine phosphorylation of PKCζ was completely abolished (Figure 3.7). This was strong evidence that Src is critically involved in the tyrosine phosphorylation of PKCζ downstream of EGFR activation. We suggested that Src activated upon EGFR stimulation might be the proximate kinase that mediates EGF-induced tyrosine phopshorylation of PKCζ.

Since PKCζ is downstream effector of PI3K in many cell systems and EGFR stimulation activates PI3K as well we assessed the influence of PI3K on EGFR-mediated PKCζ tyrosine phosphorylation. In addition, Naknishi *et al*., 1993 have shown that PKCζ is activated by PIP3. Partial decrease in EGF-induced tyrosine phosphorylation of PKCζ caused by PI3K inhibitors Wortmannin and LY294002 suggested that PI3K activity contributes to PKCζ tyrosine phosphorylation (Figure 3.8). PI3K inhibitors blocked the effect of insulin on tyrosine phosphorylation of PKCβII and ζ in primary skeletal muscle cultures (Braiman *et al*., 1999). PI3K involvement in our system might reflect the requirement for its product PIP3 in recruitment of PKCζ close to the tyrosine kinase. Using PTEN co-expression we found a decrease in EGF-induced tyrosine phosphorylation of PKCζ, but the result are not conclusive due to the significant decrease in EGFR expression level upon triple co-transfection. Nevertheless the experiments with the PI3K inhibitors demonstrated that PI3K activity is implicated to some extent in PKCζ tyrosine phosphorylation downstream of EGFR.

We determined the importance of tyrosine residues in the cytoplasmic part of EGFR for the tyrosine phosphorylation of PKCζ. Upon co-expression of Y1173F, Y992F and Y1086F EGFR mutants PKCζ tyrosine phosphorylation was even enhanced. Phospho-tyrosine 992 and phospho-tyrosine 1173 couple EGFR to phospholipase C-γ (PLC-γ) pathway, phospho-tyrosine 1086 and phospho-tyrosine 1173 represent link to activation of ERK/MAPK and phospho-tyrosine 1086 additionally contributes to activation of Akt/PKB cascade (Figure 1.1). One possible explanation for the stimulating effect on PKCζ tyrosine phosphorylation found upon expression of the respective EGFR mutants might be that the impairment of binding of respective docking proteins increases the accessibility of EGFR for PKCζ tyrosine phosphorylation machinery via release of some sterical interference. On the other hand the increase in PKCζ tyrosine phosphorylation could be explained by the involvement of at least tyrosine 1173 in recruitment of protein tyrosine phosphatase (SHP1) that promote downregulation of EGFR by dephosphorylation. This finding might either represent additional support for the interrelation of EGFR activity state and PKCζ

tyrosine phosphorylation or suggest involvement of EGFR-activated PTPs in dephosphorylation of PKCζ. In cells expressing EGFR Y845F mutant the basal as well as the EGF-induced tyrosine phosphorylation of PKCζ was clearly reduced (Figure 3.9). In contrast to the autophosphorylation sites tyrosine 845 in the activation loop of EGFR represents Src-phosphorylation site (Biscardi *et al*., 1999). Thereby our results demonstrate critical involvement of Src-phosphorylation site in the process of PKCζ tyrosine phosphorylation. Moreover in combination with the dramatic effect of dominant negative Src these findings reveal that Src activation upon EGFR stimulation and subsequent Src-dependent phosphorylation of EGFR on tyrosine 845 are necessary steps mediating EGFR-dependent tyrosine phosphorylation of PKCζ. We cannot exclude EGFR or Src family kinases as upstream tyrosine kinase mediating PKCζ tyrosine phosphorylation. Src activation in response to EGF and the following phosphorylation of tyrosine 845 could assist for the recruitment of PKCζ to the EGFR signalling complex and it can be EGFR tyrosine kinase itself that mediate the tyrosine phosphorylation of PKCζ. Thus, our results implicate PKCζ tyrosine phosphorylation of as one of the targets of the functional synergism between Src and EGFR in the cancer progression. Both c-Src and EGFR are overexpressed in high percentages of human breast cancers. C-Src potentiates the mitogenic and tumorigenic capacity of overexpressed EGFR, in part by physical association with the receptor and Src-dependent phosphorylation of tyrosine 845 and tyrosine 1101 (Biscardi *et al*., 2000). Y845F mutant of EGFR was found to retain full catalytic activity and its ability to activate ERK2, but to affect in dominant-negative fashion EGF, serum and LPA-induced DNA synthesis (Tice *et al*., 1999). The mitogenic pathway involving phosphorylation of tyrosine 845 of EGFR can be initiated by cytokine and estrogen receptors as well, is independent of ERK2 activation and might involve PI3K and ERK5. A plausible candidate for ERK-independent mitogenic effect downstream of EGFR is STAT5 whose tyrosine phosphorylation in response to EGF has been reported to be dependent in part on Src activity and the presence of Src-mediated phosphorylation of EGFR Tyr-845 (Kloth *et al*., 2003). In A431 cells Sato *et al*., 2003 demonstrated that Src phosphorylation of EGFR Tyr-845 triggers cell cycle arrest and apoptosis via upregulation of STAT-p21waf1 pathway. Recently, cytochrome c oxidase subunit II has been found to bind to phospho-tyrosine 845 on the EGFR (Boerner *et al*., 2004). Our findings suggest that PKCζ might represent another target of the synergistic signalling of Src and EGFR triggered by tyrosine phosphorylation similarly to STAT5a.

It is increasingly apparent that signal transduction involves the physical assembly of individual components of a pathway into multiprotein complex. Such a multiprotein complex that induce mitogenic signaling assembles on the prototypical receptor tyrosine kinase EGFR. Moreover, the association of PKC with respective tyrosine kinases very often accompanies the process of PKC tyrosine phosphorylation (Table 1). There are evidences for PKCδ interactions *in vitro* with Lck

(Konishi *et al*., 2001) and Src (Blake *et al*., 1999) as well as association *in vivo* with Src (Shnamugam *et al*., 1998; Song *et al*., 1998), Pyk2 (Wrenn *et al*., 2001) and Lyn (Song *et al*., 1998). PKCδ was found to be constitutively associated with Src in phosphorylation-independent manner, whereas PDGF stimulation-induced PKCδ association with Fyn was dependent on the phosphorylation of tyrosine 187 (Kronfeld *et al*., 2000). Interactions of other PKC isoforms with cytoplasmic tyrosine kinases have been also identified- PKCθ-Lck in T cells (Liu *et al*., 2000), PKCθ-Btk in platelets (Crosby and Poole, 2002), PKCθ- Src and Lyn in mast cells (Liu *et al*, 2001), PKCι-Src in PC12 cells (Wooten *et al*, 2001a). Recruitment of PKCδ to the FcεRI upon antigen stimulation has been reported (Haleem-Smith *et al*., 1995) and tyrosine-phosphorylated PKC was less associated with the receptor. PKCζ interaction with many receptor complexes has been investigated (IL-1R, TFFR, NGFR) and is mediated via PKCζ-interacting protein (p62/ZIP) (see Figure 1.11.), but up to now there are no data about the presence of PKCζ in the EGFR signalling complex.

We identified the co-immunoprecipitation of PKCζ and EGFR as an indication for either direct or indirect physical association of both proteins (Figure 3.11). Co-immunoprecipitation was detected vice versa- PKCζ in EGFR immunoprecipitates and EGFR in PKCζ immunoprecipitates upon their overexpression as well as endogenously in COS-7 cells and in MCF-7 cells (Figure 3.12). We expected that PKCζ-EGFR interaction might be present in cell line with highly overexpressed EGFR such as A431. In EGFR immunoprecipitates from A431 we detected another tyrosine-phosphorylated protein with lower molecular weight than PKCζ, which might be Src family kinase member. Only in PKCζ immunoprecipitates small amount of EGFR has been detected upon stimulation with 10nM EGF. Moreover, PKCζ was only slightly tyrosine phosphorylated in A431 in response to EGF, whereas in MCF-7 cells EGF-induced tyrosine phosphorylation of PKCζ was much more pronounced. Therefore it could be that another important mediator of the physical interaction of PKCζ and EGFR plays a limiting role in the complex assembly in A431.

The dependence of the interaction on the stoichiometric ratio of both proteins indicated that PKCζ could exert negative feedback control on EGFR function and therefore to regulate its level of tyrosine phosphorylation mediated by EGFR. Tyrosine phosphorylation of PKCζ was lowered when the ratio of both DNAs during transfection was 2:1 (PKCζ : EGFR). Moreover, the association of PKCζ with EGFR upon such ratio was higher under basal conditions and decreased in response to EGF stimulation. In contrast, when both DNAs were transfected in ratio 1:1 the interaction was lower under basal conditions and increased upon EGF stimulation. Furthermore, the same trend was valid for the tyrosine phosphorylation of EGFR in PKCζ immunoprecipitates (Figure 3.11). This observation could be an indication for increased association of the excess amount of PKCζ with the EGFR under basal conditions and PKCζ-dependent decrease in its

tyrosine phosphorylation and activity leading to decreased tyrosine phosphorylation of PKCζ. Thereby kind of novel cross talk might take place between EGFR and PKCζ with EGFR inducing PKCζ tyrosine phosphorylation and feedback regulation on EGFR tyrosine kinase activity by PKCζ. Recently, cross talk among serine/threonine and tyrosine kinases is demonstrated to involve reciprocal phosphorylation and regulation of their activities (Crosby and Poole, 2003). Additional line of evidence supporting our idea came from the experiments with PKC mutants with different activity state. The constitutively active mutants of both PKCζ and PKCε were significantly lower tyrosine phosphorylated, whereas the respective inactive mutants showed higher tyrosine phosphorylation in response to EGF stimulation (Figure 3.10.). Increased interaction of constitutively active PKCζ with EGFR under basal conditions detected by co-immunoprecipitation (Figure 3.13) probably provides the structural bases for PKC-dependent threonine phosphorylation of EGFR. PKC-dependent phosphorylation of EGFR on threonine 654 in the juxtamembrane domain is implied in inhibition of its tyrosine kinase activity (Lund *et al*., 1990). Threonine-phosphorylated EGFR molecules undergo normal internalization, but instead of sorting to lysosomal degradation, they recycle back to the cell surface. Thereby, the heterologous desensitization restrains ligand-induced down-regulation of EGFR (Bao *et al*., 2000). PMA-regulated PKCs are implicated in both rapid and long term effects on RTK phosphorylation and degradation (Seedorf *et al*., 1995). The short-term effect of decreased EGFR tyrosine phosphorylation within minutes of PMA treatment is mediated via activity of PTPs, rather than by PKC-mediated receptor phosphorylation. In contrast, sustained activation of PKCα by PMA results in stable complex with EGFR. Co-immunoprecipitation was detected in the absence of EGF and was further increased by ligand addition similarly to our observation for the association of PMA-unresponsive PKCζ with the EGFR.

The time course of the interaction of PKCζ and EGFR has been followed in vice versa co-immunoprecipitation and revealed interesting biphasic patterns. There is some constitutive association of PKCζ and EGFR under non-stimulated conditions, which increases upon EGF-stimulation very fast within the 30 sec-1 min followed by a reduced interaction accompanied with elevated threonine phosphorylation of EGFR between 1 and 5 min EGF stimulation (Figure 3.13). The interaction is restored upon prolonged EGF stimulation (15-30 min). Kronfeld I. *et al*., 2000 investigated the kinetic of PDGF-induced PKCδ interaction with Fyn. They reported fast increase in the association after 1 min treatment, which further gradually increase up to 15 min PDGF stimulation and decreased upon 30 min prolonged stimulation time. Thereby PKCδ physical association with Fyn appears to be more stable and longer lasting over the time.

Upon co-expression of EGFR dimerization mutant the co-immunoprecipitatation of EGFR and PKCζ was abrogated, thereby demonstrating that PKCζ associates with the activated receptor (Figure 3.14). Co-immunoprecipitation of PKCζ with EGFR mutants harboring mutations of

single tyrosine residue in the cytoplasmic part indicated decreased interaction with EGFR Y1086F, probably reflecting that functional activation of PI3K pathway downstream of EGFR is important for the interaction of PKCζ with the EGFR- mediated via PIP3 generation and PIP3-dependent recruitment of PKCζ to the membrane compartment, which increase the probability for the interaction with the EGFR. The interaction of EGFR Y992F with the PKCζ is to some extent more pronounced than that of the EGFR wild type indicating probably for some competition between PKCζ and PLCγ for interaction with EGFR. Moreover, the EGFR mutant harboring substitution of the PLCγ and SHP1-docking tyrosine 1173 to phenylalanine showed EGF-stimulation-dependent increase of the co-immunoprecipitation with PKCζ. This suggests that upon EGF-stimulation and in the absence of bound PLCγ and/or SHP1 the interaction of EGFR and PKCζ might be favored. In addition to its critical implication in mediating PKCζ tyrosine phosphorylation downstream of EGFR activation, phopshorylation of tyrosine 845 of EGFR is necessary for the interaction of PKCζ with the receptor. Upon co-expression of EGFR Y845F mutant the co-immunoprecipitation was clearly abrogated. Seedorf *et al*., 1995 observed association of PMA-responsive PKCα with kinase-deficient EGFR (K721A) and suggested that receptor autophosphorylation is not required for PKC binding. We found that the physical association of PMA-unresponsive PKCζ with the EGFR strongly depends on the phosphorylation of tyrosine 845 on EGFR. Moreover, the EGF-dependent increase in the interaction of PKCζ with EGFR was diminished in cells treated with PI3K inhibitors-LY294002 and Wortmannin, suggesting a role of PI3K or its product PIP3 in recruitment of PKCζ close to the receptor complex and that this especially is necessary to support the increased association upon EGF stimulation, via activation of PI3K. Gomez *et al*., 1996 demonstrated association of PI3K to PKCζ upon IL-2 stimulation. Our results show that interaction of PKCζ with 110 beta subunit of PI3K as well as with Akt takes place mostly under basal conditions and that either overexpression of EGFR or its stimulation with EGF abolished the interaction of PKCζ with these signalling proteins from PI3K/Akt pathway (Figure 3.15). The interaction of PKCζ with wild type 110 beta subunit of PI3K under basal conditions together with the decreased association of PKCζ with EGFR in cells cotransfected with wild type 110 beta might explain the decreased basal tyrosine phosphorylation of PKCζ. We can speculate that under non-stimulated conditions PKCζ is sequestered in the cytoplasm by interactions with PI3K beta and Akt, whereas upon EGF stimulation PKCζ interaction with EGFR is favored.

Very important for the appropriate PKC function is its targeting via protein-lipid and protein-protein interactions to specific subcellular compartments, close to its activators and substrates. Membrane translocation of PKC is considered as indicative for its activation. In some cases tyrosine phosphorylation of PKC is accompanied with membrane translocation- PKCδ and θ upon antigen stimulation of FcεRI (Haleem-Smith *et al*., 1995; Liu *et al*., 2001) and PKCδ upon

BCR activation (Barbazuk and Gold, 1999). It has been suggested that the tyrosine kinase, which is involved, is membrane localized since the translocation is required for tyrosine phosphorylation of PKCδ, rather than the membrane translocation being dependent on PKCδ tyrosine phosphorylation. On the other hand, there are many examples for tyrosine phosphorylation of PKC without significant membrane translocation – upon hydrogen peroxide treatment (Konishi *et al*., 1997) and in response to EGFR stimulation in keratinocytes (Denning *et al*., 1993). PKCδ tyrosine phosphorylation has been shown to occur simultaneously with Golgi translocation in response to IFN-γ (Kajimoto *et al*., 2001), or to be related to nuclear translocation during etoposide-induced apoptosis (Blass et al., 2002). Tyrosine phosphorylation of PKCθ and translocation to the immunological synapse between T cell and antigen presenting cell has been demonstrated (Liu *et al*., 2000). NGF-induced tyrosine phosphorylation of PKCλ/ι regulates its localization in a complex with p62/ZIP in the late endosomes (Samuels *et al*., 2001) or facilitated its nuclear import dependent on the phosphorylation of tyrosine 256 (White *et al*., 2002). A role of tyrosine kinases as RACKs (Fyn for PKCθ in T cells) has been discussed (Ron *et al*., 1999). Growth factors (e.g. EGF) activate PKC in several cell types including the epithelium as evidenced by increased amount of PKC found in the membrane fraction. EGF caused translocation of both PKCα and βI into membranes of canine gastric cells (Wang *et al*., 1996) and membrane association of PKCα in mammary epithelial cultures (Birkenfeld *et al*., 1996). EGF treatment has been shown to cause redistribution of PKCα, βI and ζ to membrane fractions in human intestinal cell line Caco-2 (Banan *et al*., 2001). Recently, Sun *et al*., 2005 have found EGF-induced translocation of PKCζ from the cytosol to the plasma membrane in human breast cancer cells.

There are contradictory data about the subcellular localization patterns of PKCζ. Diffuse cytosolic staining for ectopically expressed PKCζ has been observed (Goodnight *et al*., 1995; Puls *et al*., 1997). On the other hand, endogenous PKCζ showed a vesicular punctate pattern of localization (Westermann *et al*., 1996). Sanchez *et al*., 1998 demonstrated punctate vesicular pattern of subcellular localization for either ectopically expressed or endogenous aPKC isoforms - PKCλ/ι and PKCζ and p62 in HeLa cells. The constitutive colocalization of p62 with aPKCs has not changed in response to EGF stimulation. Furthermore, p62 colocalizes with the internalized EGFR in the lysosome-targeted late endosomes. The authors proposed that atypical PKCs anchored by p62 in the lysosome-targeted endosomal compartment are involved in the control of the growth factor receptor trafficking. Similar endosomal colocalization of aPKC, p62/ZIP and the internalized NGFR was shown to contribute to MEK5 activation during NGF-mediated differentiation process (Samuels *et al*., 2001; Geetha *et al*., 2003).

Using confocal microscopy we observed membrane translocation of PKCζ upon EGF stimlation and its colocalization with EGFR (Figure 3.17). Under unstimulated conditions we found diffuse

subcellular localization of PKCζ with some concentration in the perinuclear area in COS-7 cells expressing PKCζ-EYFP. Upon 1 min stimulation with 10nM EGF we detected membrane translocation of PKCζ. In cells co-expressing EGFR-CFP and PKCζ-EYFP we identified that EGFR and PKCζ colocalize to some extent in the perinuclear region under basal conditions and more pronounced at the plasma membrane upon EGF stimulation. Whereas the colocalization without cell stimulation might reflect the transport of both overexpressed proteins through Golgi, the membrane colocalization of EGFR and PKCζ is indication for recruitment of PKCζ to the EGFR signalling complex at the membrane, localization, which is required for EGFR-mediated tyrosine phosphorylation of PKCζ. It was not our aim to investigate whether PKCζ colocalize with EGFR during receptor internalization.

Concerning the mechanism of association of PKCζ with the EGFR we can give some ideas based on the known interactions. ZIP/p62 association to TrkA has been mapped to the juxtamembrane region (amino acids 472-493) (Wooten *et al*., 2001a). Sequence comparison revealed that the motif involved in p62/ZIP binding to TrkA is highly conserved in EGFR (amino acids 710-732), thus suggesting for possible interaction.

(TrkA) LGEGAFG
(EGFR) LGSGAFG

ZIP/p62 may bind to the EGFR also indirectly via receptor-interacting protein (RIP), since EGFR has been found to ineract with RIP (Habib *et al*., 2001) and on the other hand RIP association with ZIP/p62 has been demonstrated in context of TNFR (Sanz *et al*., 1999). Another possible link between the EGFR and PKCζ might be provided by tyrosine-containing tight turn structures present in the endocytic codes of many receptors. EGFR synthetic code NFAYF (native QQGFF) was recognized in two-hybrid system by LIM domains of PKC-interacting partner ENH (Enigma homolog) (Wu and Gill *et al*., 1994; Kuroda *et al*., 1996).

Whereas modification of PKC by serine/threonine phopshorylation involves highly conserved residues in the catalytic domain, various tyrosine residues in the regulatory and the catalytic domain are targets for phosphorylation in PKC isoform- and stimulus-specific manner. Conserved among all members of the PKC family are PKC tyrosine residues 451, 469, 512, 523 triggered by H_2O_2-induced tyrosine phosphorylation of PKCδ, but none of them is phosphorylated by Src within atypical PKCι. Hydrogen peroxide induces phosphorylation of tyrosines located in the catalytic domain (Konishi *et al*., 2001), Src is involved in phosphorylation of tyrosine-311 at the hinge region (Blake *et al*., 1999), whereas many stimuli target PKCδ tyrosine residues in the regulatory domain- tyrosine-52 upon engagement of IgE receptor (Szallasi *et al*, 1995), tyrosine-187 following PMA and PDGF stimulation in NIH 3T3 (Li *et al*, 1996; Szallasi *et al*., 1995) and C6 cells (Kronfeld *et al*., 2000), tyrosine-155-upon

PMA stimulation of C6 cells (Kronfeld *et al*., 2000). Isoform-specific phosphorylation sites were identified for other PKC isoforms as well. Lck in involved in phosphorylation of PKCθ at tyrosine-90 during T cell activation (Liu *et al*., 2000). NGF- stimulated multisite tyrosine phosphorylation of atypical PKCι has been shown to involve three residues within the catalytic domain- tyrosines 256, 271 and 325 (Wooten *et al*., 2001a). It seems that modification triggers sets of tyrosine residues rather than only one. For example, Kronfeld *et al*., 2000 reported that only after substitution of five tyrosines in the regulatory domain with phenylalanine (PKCδ5 -52, 64, 155, 187 and 565) there is no significant degree of enhanced tyrosine phosphorylation in response to PMA and PDGF.

We identified tyrosine 417 in the catalytic domain of PKCζ as one of the phosphorylation sites in response to EGFR stimulation. The tyrosine phosphorylation of PKCζ Y417F was partly reduced compared to wild type PKCζ, demonstrating that there are still other tyrosines targeted by EGF-induced phosphorylation within PKCζ (Figure 3.18). Intrestingly, tyrosine 417 is located in the vicinity of the activation loop threonine residue, embedded in highly conserved region of the PKC catalytic domain and is conserved among all members of PKC family as well as in PKA, PKBα/Akt1 and p70S6K. The corresponding tyrosine 512 within PKCδ is one of residues phosphorylated upon hydrogen peroxide treatment (Konishi *et al*., 1997).

Tyrosine phosphorylation of PKC isoforms may have quite different effects on PKC activity ranging from inhibition of PKCδ upon Ras-transformation and in response to EGFR stimulation in keratinocytes (Denning *et al*., 1996) up to constitutively active DAG-independent PKCδ upon hydrogen peroxide-induced tyrosine phosphorylation in the catalytic domain (Konishi *et al*., 1997). Phosphorylation of some tyrosines (e.g. tyrosine 187 of PKCδ, Li *et al*., 1996) has no influence on PKC activity. Similarly, Kronfeld I. et al, 2000 showed that single substitution of tyrosine-52, 155 or 187 as well as of all five (in PKCδ5 mutant) did not change enzyme activity. Tyrosine phosphorylation could alter the substrate specificity as well. Tyrosine-phosphorylated PKCδ in response to IgE receptor engagement had diminished activity toward the receptor γ-chain peptide as a substrate (Haleem-Smith *et al*., 1995).

PKCλ activation has been found to occur through PI3K upon EGF and PDGF stimulation (Akimoto *et al*., 1996). In our expression model PKCζ wild type was activated in response to EGFR stimulation (Figure 3.19). The EGF-induced PKCζ activity is around 180% in relation to the basal activity, but is significantly lower compared to PKCζ activation caused by hydrogen peroxide treatment. Moreover, the activity of PKCζ correlated with its tyrosine phosphorylation state. Inhibitors affecting the tyrosine phosphorylation (AG1478 and LY294002) were diminishing PKCζ activation as well (Figure 3.20). Chou *et al*., 1998, have reported the activation of PKCζ downstream of EGFR activation and its complete dependency on PI3K and PDK1 mediated phosphorylation at the activation loop. The crucial role of the phoshorylation of

threonine 410 at the activation loop for the catalytic activity of PKC is well established. The negative charge at the AL site aligns correctly the residues for catalysis and unmasks the entrance to the substrate-binding cavity, thereby being absolutely required for the PKC maturation (Orr and Newton, 1994). Fully functional, mature enzyme results from mimicking this negative charge by substitution of Thr for Glu, whereas mutant with unphosphorytable Ala instead of Thr is inactive (Cazaubon *et al*., 1994). PKCδ phosphorylation of Thr in the activation loop is not necessary because a five residues upstream positioned Glu partially fulfill the requirement for negative charge (Stempka *et al*., 1997). The activity of PKCζ catalytic domain depends on transphosphorylation at the activation loop (Smith and Smith, 2002). We found that PKCζ Y417F mutant was catalytically inactive, similarly to PKCζ T410A mutant, showing absence of basal activity and no activation upon EGF stimulation. Only a slight increase of the activity of the mutant was observed in response to hydrogen peroxide treatment. Neither hydrogen peroxide nor EGF induced further autophosphorylation of the PKCζ Y417F. The absence of catalytic activity was accompanied with only a slight decrease in the tyrosine phosphorylation induced by both stimuli (Figure 3.21 and 3.22). Thus, tyrosine 417 of PKCζ is triggered by hydrogen peroxide-dependent phosphorylation similarly to the corresponding tyrosine 512 of PKCδ. PKCδ Y512F showed attenuated activation in response to hydrogen peroxide but the same properties as the wild type enzyme upon in vitro phosphorylation by Lck (Konishi et al., 1997, 2001). Our results confirm the role of the respective tyrosine 417 of PKCζ in hydrogen peroxide-induced activation. Our results provide first evidence that tyrosine 417 of PKCζ is involved in EGFR-mediated activation. Moreover, a direct comparison of PKCζ Y417F and PKCζ T410A revealed that both threonine phosphorylation at the activation loop and tyrosine phosphorylation in its vicinity are required for PKCζ activity in response to EGFR. T410A mutant has intact tyrosine-417 and respectively Y417F mutant has intact threonine 410, but neither threonine-410 phosphorylation nor tyrosine-417 phosphorylation alone is sufficient for the catalytic activation. This observation suggests that similarly to the MAPK activation, EGF-induced PKCζ activation involves both threonine and tyrosine phosphorylation.

Many PKC functions in various cellular processes are influenced by its tyrosine phosphorylation. Tyrosine phosphorylation can affect protein functions either by change in the enzyme activity, or via creating new binding sites for proteins containing phospho-tyrosine binding domains. Phosphorylation of PKCα and βI upon FcεRI activation on tyrosines neighboring serine residues at the turn motif creates docking sites for Grb2 and contributes to the activation of Ras/ERK cascade (Kawakami *et al*., 2003). Role of PKCθ tyrosine phosphorylation during T cell (Liu *et al*., 2001) and platelets (Crosby and Poole, 2002) activation has been proposed as well as importance of PKCδ tyrosine phosphorylation in mast cells degranulation (Haleem-Smith *et al*., 1995) and for the process of GPCR agonists-induced

fluid secretion from the salivary gland (Soltoff *et al*., 1995). PKC tyrosine phosphorylation can contribute to apoptosis induction. Phosphorylation of tyrosines in the hinge region surrounding caspase-cleavage site of PKCδ might contribute to oxidative stress-induced apoptosis (Konishi et al., 1999) and phosphorylation at tyrosine 311 is shown to promote PKCδ degradation (Blake *et al*., 1999). Tyrosine phosphorylation and activation of PKCδ by UV in HaCaT human keratinocytes is implicated in induction of apoptosis (Fukunaga *et al*., 2001). Etoposide-induced apoptosis depends on tyrosine phosphorylation-dependent PKCδ cleavage by caspases (Blass *et al*., 2002). Tyrosine phosphorylation of PKC is related to the differentiation of many cell types as well. Positive influence of tyrosine phosphorylated PKCλ/ι in NGF-induced neurite differentiation (Wooten *et al*., 2001) and of PKCδ in TPA induced monocytic differentiation of 32D hematopietic cells (Li *et al*., 1994) has been demonstrated. PKCδ is critically involved in the process of Ca^{2+}-induced keratinocyte differentiation (Denning *et al*., 2000). Its inactivation via EGFR-mediated tyrosine phosphorylation serves to turn off the differentiation program at last stages of normal differentiation process and to block differentiation program during neoplastic transformation. Tyrosine phosphorylation of PKCδ on tyrosine 155 upon PE stimulation of NIH3T3 plays a role in the control of the growth rate and of density at confluency, with tumorigenic properties of Y155F mutant (Acs *et al*., 2000). Similarly, phosphorylation of PKCδ on tyrosine 155 by PE in C6 glioma cells is critical for inhibition of proliferation. In contrast phosphorylation of tyrosine-187 in reponse to PMA has different outcome concerning the differentiation in different cellular context- inducing monocytic differentition or inhibition of differentiation of glioma cells. Kronfeld I. *et al*, 2000 proposed that tyrosine phosphorylation of PKC on different tyrosine residues might represent a point of divergence in its functions. It has been demonstrated in C6 glioma cells that phosphorylation of different tyrosines mediate different functions of PKCδ with Y155 mediating PKCδ inhibitory effect on cell proliferation whereas Y187 mediates PKCδ inhibitory effect on GS astrocyte differentiation marker expression.

MEK5 represents one known target of PKCζ during EGF mitogenic signalling, whose activation is independent on PKCζ enzymatic activity (Diaz-Meco and Moscat 2001). We found that expression of wild type PKCζ leads to increased EGF-induced ERK/MAPK activity, indicating that PKCζ is involved in ERK/MAPK cascade activated downstream of EGFR (Figure 3.23). The role of PKCζ in ERK/MAPK cascade has been demonstrated in many cell systems (see Intoduction). PKCζ function as MEK1 kinase in this signalling pathway has been suggested. Indeed PKCζ associates with MEK1, but it is still unclear whether PKCζ phosphorylates MEK1 directly (Monick *et al*., 2000). Our results reveal that the catalytic activation of PKCζ upon EGFR stimulation is of importance for ERK/MAPK pathway. In response to stimulation with low ligand concentration (0.1nM EGF) there was a significant

increase in MAPK activity in cells expressing PKCζ WT. This effect on MAPK activity was lost when instead of wild type either PKCζ Y417F or PKCζ T410A were transfected.

Another EGFR activation-triggered signalling pathway in which PKCζ could take part involves pro-survival Akt/PKB. Both PKCζ and Akt/PKB are activated downstream of PI3K activation in response to EGFR stimulation via PDK1-dependent phosphorylation. PKCζ is known as an adaptor for PKBγ/AKT3 phosphorylation by PDK2 (Hodgkinson *et al*., 2002). Interaction of PKCζ and PKBα/AKT1 has been reported in EGFR-overexpressing human breast carcinoma cell line and PKCζ-dependent decrease in Akt phosphorylation/activity was observed (Mao *et al*., 2000). There are no reports for PKCζ-mediated Akt1/PKBα phosphorylation. In our experiments EGF stimulation in COS-7 induced transient Akt activation as indicated by phosphorylation of serine-473. In cells expressing PKCζ EGF-dependent Akt activation was not significantly affected, whereas in cells co-transfected with PKCζ along with EGFR Akt activation upon EGF stimulation was reduced. Akt activation might involve PKCζ kinase activity and/or tyrosine phosphorylation, since in cells co-transfected with PKCζ Y417F along with EGFR, the increase in Akt phosphorylation in response to 0.1nM EGF was diminished (Figure 3.24). The reduction in EGF-induced Akt activation upon co-expression of PKCζ and EGFR might have another mechanism, not related to PKCζ activation by tyrosine phosphorylation.

The transcription factor nuclear factor-κB (NF- κB) is activated by a diverse number of stimuli including TNFα, IL-1, UV irradiation, viruses, as well as receptor-tyrosine kinases such as EGFR. Downstream of TNFR, IL-1R or NGFR PKCζ functions as IκB kinase (IKK) (Hirai and Chida, 2003). Activated by PKCζ-dependent phosphorylation IKK phosphorylates IκB, targeting it for ubiquitination and proteasome degradation and thereby NF-κB is released from cytoplasmic sequestration, translocates to the nucleus and activates gene expression. Recently, it has been found that PKCζ may contribute to NF-κB activation directly by phosphorylation of Ser311 of RelA (Duran *et al*., 2003). EGFR has been shown to activate NF-κB slowly in aortic smooth muscle cells, more robust in A431 cell, transiently in mouse embryo fibroblasts (Sun and Carpenter, 1998) and in several EGFR-overexpressing breast cancer cell lines. Downstream of growth factors stimulation MAPK/ERK cascade via NAK activation as well as SAPK/JNK cascade induces phosphorylation and activation of IKK. PKCζ could represent another EGFR target that act as IKK kinase. A novel EGFR multiprotein complex termed signalosome has been identified to involve RIP and NF-κB-inducing kinase (NIK), which mediates NF- κB induction via I-κBα phosphorylation on serines 32 and 36 (Habib *et al*., 2001). Moreover, high level of EGFR overexpression, a frequent occurrence in human tumors, is optimal for EGFR-induced NF- κB activation. There are controversial data about the role of PKCζ in NF-κB activation downstream of EGFR. Banan A. et al, 2003 have reported that epidermal growth

factor protects intestinal Caco-2 cells from oxidant-induced NF-κB activation by PKCζ activation via enhanced stability of I-κBα due to reduced phosphorylation. These observations provoked us to analyse PKCζ function in I-κBα phosphorylation downstream of EGFR. EGF-stimulation in COS-7 cells led to increased phosphorylation of I-κBα, indicative for NF- κB activation. PKCζ as well as EGFR overexpression caused increased I-κBα phosphorylation. In cells expressing PKCζ Y417F mutant I-κBα phosphorylation was clearly reduced (Figure 3.24). This finding supports the suggestion that the catalytic activity of PKCζ is implicated in I-κBα phosphorylation and thereby in NF- κB activation downstream of EGFR. Similarly to the Akt activation I-κBα phosphorylation was diminished upon stimulation with high EGF concentration in cells co-transected with PKCζ along with EGFR. This could be some preventive cross-talk mechanism, which avoid increased Akt and NF- κB activation.

The influence of PKCζ Y417F mutant on EGFR-activated ERK/MAPK, PKB/Akt and NF- κB pathways reveals involvement of PKCζ catalytic activity and/or tyrosine phosphorylation and that EGFR-mediated PKCζ phosphorylation on tyrosine 417 is necessary for PKCζ function downstream of EGFR.

There are contradictory results concerning the pro-apoptotic, as well as pro-survival role of PKCζ (see introduction). We found that neither PKCζ nor EGFR expression cause PARP cleavage in unstimulated cells as well as upon EGF stimulation. Moreover, PKCζ catalytic activation and tyrosine phosphorylation are implicated in the EGFR-activated signalling cascades with proliferatve and pro-survival functions. Therefore we assessed the effect of PKCζ on cell proliferation. [^{3}H]-thymidine incorporation assay demonstrated that cells overexpressing wild type PKCζ have increased basal proliferation level, which is not further increased upon EGF stimulation. Similarly, Le Good *et al*., 2004 have identified enhanced proliferation of Rat2 fibroblasts over-expressing wild type PKCζ. Moreover, expressed PKCζ activity was paralleled by cell transformation. In contrast, Montaner *et al*., 1995 have shown that PKCζ does not induce cell transformation in fibroblasts. Very recently, a role of PKCζ in cancer cell metastasis via modulation of actin cytoskeleton structure has been suggested. PKCζ has been identified as an essential component of the EGF-stimulated chemotaxis in human breast cancer cells (Sun *et al*., 2005). PKCζ is implicated in estradiol-triggered G1/S progression of MCF-7 cells (Castoria *et al*., 2004). Activated in PI3K-dependent manner PKCζ recruits Ras to a multimolecular complex that consists of estrogen receptor, src, PI3K and facilitates Src-dependent Ras activation. We obtained evidence that proliferative, pro-survival function of PKCζ requires its catalytic activity and/or tyrosine phosphorylation since upon transfection of PKCζ Y417F mutant no increase in the basal rate of DNA synthesis was observed and EGF-stimulated DNA synthesis was also clearly diminished (Figure 3.25).

PKC isoforms involvement in GPCR signaling toward ERK/MAPK activation might occur at many levels: in GPCR-mediated ERK/MAPK activation independent of RTK transactivation, in GPCR-induced transactivation of EGFR and in downstream signalling of transactivated EGFR as well. For example, recently Buteau *et al*., 2003 have demonstrated the involvement of PKCζ in glucagon-like peptide-induced pancreatic beta-cell proliferation downstream of EGFR transactivation. On the other hand, tyrosine phosphorylation of PKCδ upon GPCR stimulation has been already demonstrated in acinar cells in response to carbachol and substance P (Soltoff *et al*., 1995; Wrenn, 2001) and upon cholecystokinin (CCK) stimulation (Tapia *et al*., 2002). We identified that tyrosine phosphorylation of PKCζ occurs upon stimulation of two endogenously expressed GPCRs in COS-7 cells - lysophosphatidic acid receptor and β2-adrenergic receptor with LPA and isoproterenol respectively (Figure 3.26). Lysophosphatidic acid (LPA) is a mitogenic phospholipid messenger that binds to GPCR family receptor and mediates Gi-dependent inhibition of adenylyl cyclase (AC). Involvement of PKCζ activation in LPA signalling has been already reported to mediate increase in type II AC activity in macrophages (Lin *et al*., 1999), mitogenesis in vascular smooth muscle cells (Seewald *et al*., 1999) and Ras and Raf-independent MAPK activation in CHO cells that overexpress a dominant-negative mutant of Sos (Takeda *et al*., 1999). Dual pathway of LPA-induced stimulation of ERK/MAPK was described- via EGFR transactivation and via PI3-K (Daub *et al*., 1997). LPA induced transactivation of EGFR is mediated via HB-EGF release (Prenzel *et al*., 1999). Beta2-adrenergic receptor-mediated ERK/MAPK activation involves EGFR transactivation via assembly of a multi-receptor complex with the EGFR (Maudsley *et al*., 2000), but is independent of HB-EGF release.

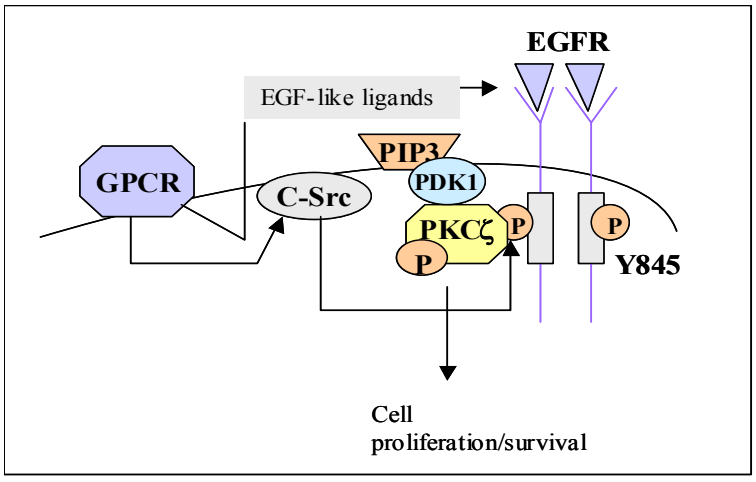


Figure 4.1. Model depicting tyrosine phosphorylation of PKCζ downstream of ligand-activated and transactivated EGFR. Shown is the critical involvement of c-Src and Src-phosphorylation site of EGFR and the requirement for PIP3 at the plasma membrane for initial recruitement of PKCζ to the membrane compartment
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We observed that in COS-7 cells both LPA and Iso induce Erk1/2 activation (Figure 3.26). Moreover both stimuli mediate EGFR transactivation as demontsrated by LPA and Iso-induced tyrosine phosphorylation of EGFR (Figure 3.27). Iso-induced tyrosine phosphorylation of PKCζ

was sensitive to pretreatment with AG1478, which represents evidence that Iso mediates tyrosine phosphorylation of PKCζ via EGFR transactivation. Both GPCR ligands not only mediate PKCζ tyrosine phosphorylation but also caused increased physical association of EGFR with PKCζ as detected by co-immunoprecipitation. Furthermore, we observed that PKCζ tyrosine-417 is the major phosphorylation site triggered by transactivated EGFR whereas it is one of the phosphorylated tyrosines downstream of ligand-activated EGFR (Figure 3.27). We found PKCζ activation upon LPA or Iso stimulation. Moreover, the activation involves tyrosine-417 phosphorylation since both LPA and Iso cannot activate PKCζ Y417F mutant (Figure 3.28). These results indicate that GPCRs also mediate tyrosine phosphorylation and activation of PKCζ via their transactivation pathways. This cannot rule out in general the potential contribution of other GPCR-activated pathways independent on EGFR transactivation to the tyrosine phosphorylation and activation of PKCζ.

In the cellular signalling network signal convergence at the level of EGFR (ligand-activated as well as transactivated via GPCRs) involves PKCζ activation and tyrosine phosphorylation at the EGFR signalling module (Figure 4.1.). PKCζ is recruited to the plasma membrane upon EGFR stimulation partly via PIP3 generation and there physically associates with EGFR in dependence on Src-phosphorylation site on EGFR- tyrosine-845. Critical role of Src in PKCζ tyrosine phosphorylation suggests that PKCζ might represent another target of the synergism between EGFR and Src implicated in the control of cell proliferation.

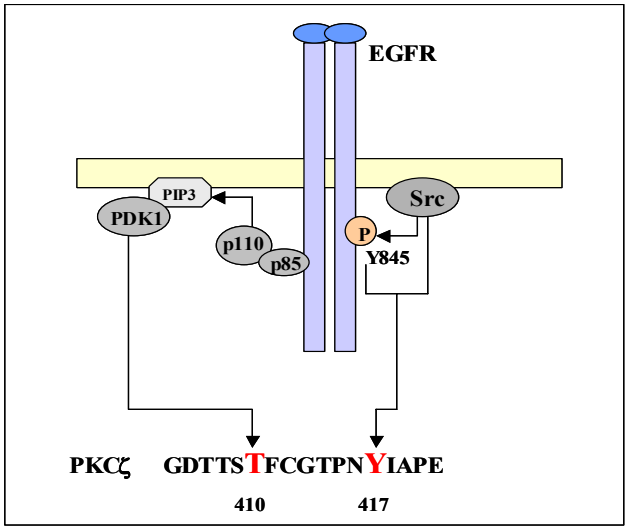

Figure 4.2. Activation of PKCζ downstream of EGFR. Shown is the involvement of both PDK-1 mediated threonine phosphorylation at the activation loop and tyrosine phosphorylation in its vicinity dependent on EGFR and Src kinase activities as well as on EGFR phospho-tyrosine 845.

Interestingly, PKCζ activation downstream of EGFR involves both PI3K/PDK1 mediated phosphorylation of threonine 410 in the activation loop as well as phosphorylation of the tyrosine 417 in the activation loop vicinity dependent on EGFR, Src and Src-phosphorylation site on EGFR (Figure 4.2.). This represents a novel paradigm for the mechanism of PKCζ activation.

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APPENDIX

Abbreviations

AC	adenylate cyclase
AKAP	A-kinase-anchoring protein
AL	activation loop
aPKC	atypical PKC
ATP	adenosine-triphosphate
β2AR	beta 2-adrenergic receptor
BMK1	big MAP kinase
Btk	Bruton's tyrosine kinase
CaMK	calcium/calmodulin-activated protein kinase
CD	catalytic domain
cPKC	clasical PKC
CRIB	Cdc42- and Rac-interacting binding
DAG	1,2- diacylglycerol
DEAE	diethylaminoethyl
ECFP	enhanced cyan fluorescent protein
ECL	enhanced chemiluminescence
EGF	epidermal growth factor receptor
EGF(R)	epidermal growth factor (receptor)
ERK	extracellular signal-regulated kinase
EYFP	enhanced yellow fluorescent protein
FKHR1	Forkhead transcription factor1
Gab	Grb-associated binder
GPCR(s)	G protein-coupled receptor(s)
Grb2	growth-factor-receptor-bound protein 2
GSK-3	glycogen synthase kinase-3
HB-EGF	heparin-binding epidermal growth factor
HM	hydrophobic motif
IFN	interferon
IgG	immunoglobulin
IKK	inhibitor of κB kinase
IL	interleukin
Ins(1,4,5)P_3	inositol-(1,4,5)- triphosphate
IP	immunoprecipitation
IR	insulin receptor
Iso	isoproterenol
IκBα	inhibitor of κB
JNK	c-jun N-terminal kinase
LIP	lambda-interacting protein
LPA(R)	lysophosphatidic acid (receptor)
MAPK	mitogen-activated protein kinase
MARCKS	myristoylated alanine-rich C kinase substrate
MBP	myelin basic protein
MEK	mitogen-activated protein kinase kinase
mTOR	mammalian target of rapamycin
NAK	NF-κB-activating kinase
NF-κB	nuclear factor-kappa B
NGF(R)	nerve growth factor (receptor)
NIK	NF-κB-inducing kinase
nPKC	novel PKC
PAR-3/ASIP	partitioning defective gene-3 product/aPKC-specific interacting protein
Par-4	prostate apoptosis response-4

PAR-6	partitioning defective gene-6 product
PB1	(Phox and Bem1p) domain
PBS	phosphate-buffered saline
PDGFR	platelet-derived growth factor receptor
PDK1	phosphoinositide-dependent kinase 1
PE	phorbol ester
PH	plecstrin homology
Pi-Thr/Tyr/Ser	phospho-Thr/Tyr/Ser
PI3K	phosphoinositide-3-kinase
PIP3	phosphatidylinositole-(3,4,5)-triphosphate
PKA	protein kinase A
PKB	protein kinase B
PKB	protein kinase B
PKC	protein kinase C
PLA_2	phospholipase A2
PLCβ/γ	phospholipase C β/γ
PLD	phospholilase D
PMA	phorbol 12-myristate 13-acetate
PRK	protein kinase C-related kinase
PS	pseudosubstrate
PTB	phosphotyrosine-binding domain
$PtdIns(3,4)P_2$	phosphatidylinositol-(3,4)- biphosphate
$PtdIns(3,4,5)P_3$	phosphatidylinositole-(3,4,5)-triphosphate
$PtdIns(4,5)P_2$	phosphatidylinositol-(4,5)-biphosphate
PtdSer	phosphatidylserine
PTEN	phosphatase and tensin homologue
PTP(s)	protein tyrosine phosphatase (s)
PY	phosphotyrosine
Pyk-2	calcium-dependent tyrosine kinase 2
RACKs	receptors for activated C kinase
RIP	receptor-interacting protein
ROS	reactive oxigen species
RTK(s)	receptor tyrosine kinase(s)
SAPK	stress-activated protein kinase
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
Shc	Src homology and collagen protein
Sos	son-of-sevenless
STAT	signal transducers and activators of transcription
STICKs	substartes that interact with C kinase
TBS	Tris-buffered saline
TCR	T cell receptor
TGFα	transforming growth factor alpha
TM	turn motif
TNF(R)	tumor necrosis factor receptor
WT	wild type
WB	Western blot
ZIP	zeta-interacting protein

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Selbständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe.

Jena, den 4 April 2005

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Erklärung zur Bewerbung

Hiermit erkläre ich, dass ich mich mit der vorliegenden Arbeit an keiner anderen Hochschule um den akademischen Grad Doctor rerum naturalium beworben habe und dass ich weder früher noch gegenwärtig die Eröffnung eines Verfahrens zum Erwerb des o.g. akademischen Grades an einer anderen Hochschule beantragt habe.

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